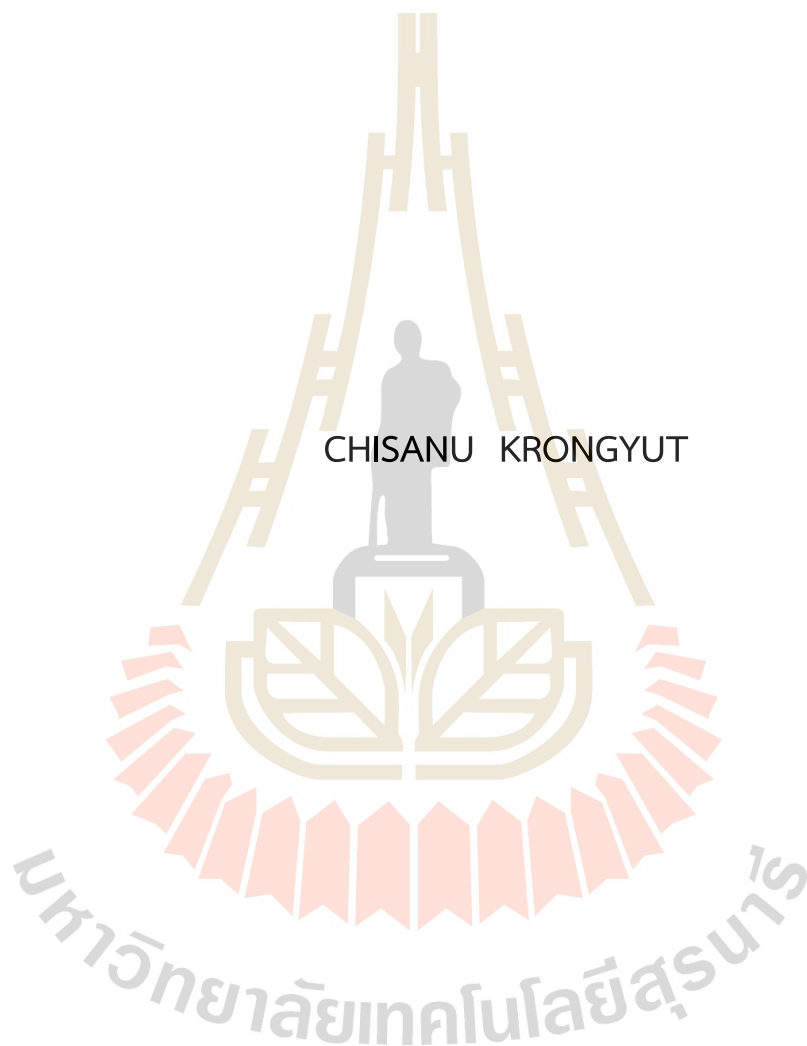


CHEMOENZYMATIC CYCLIZATION BY VANADIUM
CHLOROPEROXIDASE FOR SYNTHESIS OF
4-HYDROXYISOCHROMAN-1-ONES



CHISANU KRONGYUT

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biochemistry and Biochemical Technology
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ปฏิกิริยาเคมีเอ็นไซม์โดยวานาเดียมคลอโรเปอร์ออกซิเดสสำหรับ
การสังเคราะห์ 4-HYDROXYISOCHROMAN-1-ONES



นายชิษณุ ครองยุทธ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FOR SYNTHESIS OF 4-HYDROXYISOCROMAN-1-ONES

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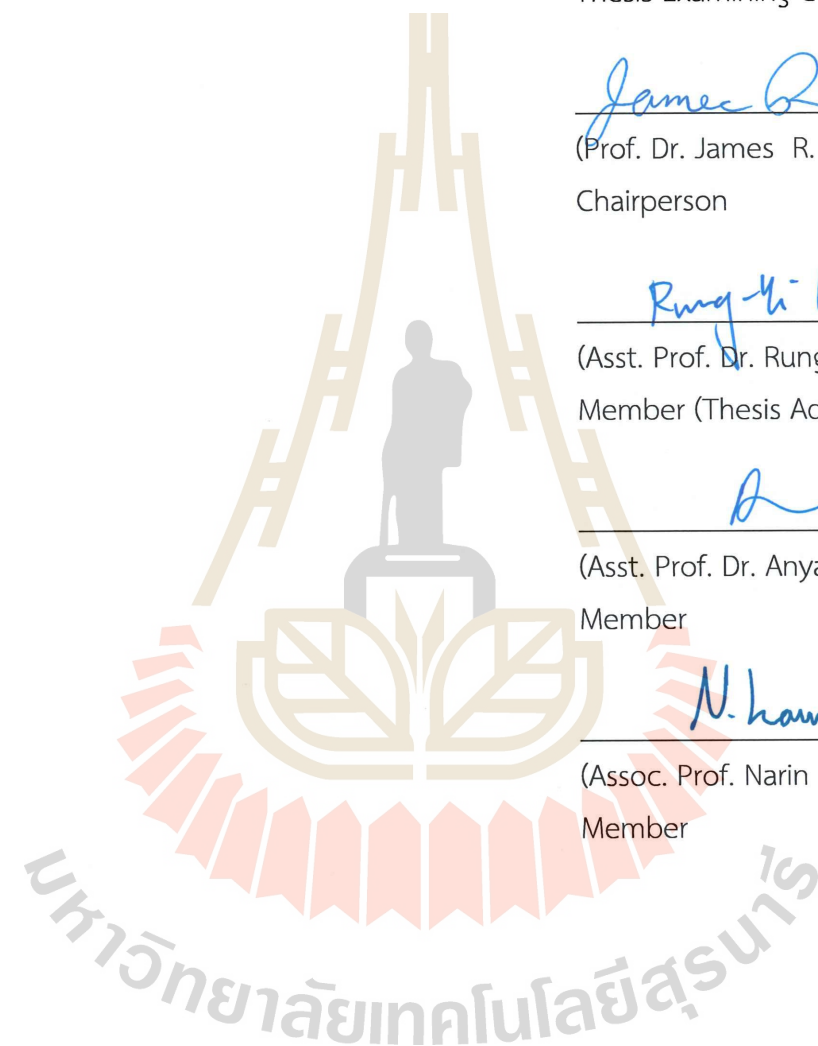
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คำสำคัญ: 4-ไฮดรอกซีไอโซโครแมน-1-วัน; วานาเดียมคลอโรเปอร์ออกซิเดส; ปฏิกริยาไซโคลเซชัน; ตัวเร่งปฏิกิริยาชีวภาพ

4-ไฮดรอกซีไอโซโครแมน-1-วัน เป็นกลุ่มของสารเมแทบอลิต์ทุติยภูมิที่จัดอยู่ในกลุ่ม 3, 4-ไดไฮดรอกซีไอโซคูมาริน ซึ่งเป็นที่รู้จักกันดีในด้านกิจกรรมทางชีวภาพที่หลากหลาย เช่น การต้านเชื้อแบคทีเรีย เชื้อรา และมะเร็ง โดยทั่วไปแล้ว การสังเคราะห์สารเหล่านี้ทำได้ผ่านกระบวนการไซโคลเซชัน โดยใช้สารประกอบไฮโดรเจนเปอร์ออกไซด์หรือ เอ็น-โบรมอสัคซินิไมด์ (NBS) ตามด้วยการไฮโดรไลซิส อย่างไรก็ตาม วิธีการเหล่านี้มักต้องใช้สภาวะที่รุนแรงและก่อให้เกิดผลพลอยได้ที่เป็นพิษ ซึ่งเป็นข้อจำกัดในทางปฏิบัติ ในการศึกษาครั้งนี้ ได้ใช้เอนไซม์ *Curvularia inaequalis* vanadium chloroperoxidase (CVCPO) เป็นตัวเร่งปฏิกิริยาเคมีเอนไซม์สำหรับการไซโคลเซชันของกรด 2-ไวนิลเบนโซอิกที่มีหมู่ให้อิเล็กตรอนต่างกัน (1a-1e) โดยใช้ KBr และ H₂O₂ ในบัฟเฟอร์ซิเตรต (pH 5) ซึ่งช่วยให้เกิดไฮโปโบรไมต์ ซึ่งเป็นสารกลางสำคัญในกระบวนการไซโคลไลเซชัน วิธีการนี้ทำให้ได้ 4-ไฮดรอกซีไอโซโครแมน-1-วัน (3a-3e) ในปริมาณสูง นอกจากนี้ เพื่อเพิ่มการละลายของสารตั้งต้นโดยไม่กระทบต่อกิจกรรมของเอนไซม์ โดยได้เติม DMSO 30% ลงในส่วนผสมของปฏิกิริยา วิธีการสังเคราะห์แบบเคมีเอนไซม์นี้ไม่เพียงทำให้กระบวนการสังเคราะห์ง่ายขึ้น แต่ยังเป็นทางเลือกที่เป็นมิตรต่อสิ่งแวดล้อมมากกว่าวิธีการทางเคมีแบบเดิม ด้วยเหตุนี้ วิธีการดังกล่าวจึงเป็นกลยุทธ์ที่ยั่งยืนและมีประสิทธิภาพสำหรับการสังเคราะห์ 4-ไฮดรอกซีไอโซโครแมน-1-วัน ซึ่งนับเป็นก้าวสำคัญในการพัฒนาวิธีการสังเคราะห์สารชีวภาพที่ปลอดภัยและเป็นมิตรต่อสิ่งแวดล้อมมากขึ้น

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CHISANU KRONHYUT : CHEMOENZYMATIC CYCLIZATION BY VANADIUM
CHLOROPEROXIDASE FOR SYNTHESIS OF 4-HYDROXYISOCHROMAN-1-ONES.
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4-Hydroxyisochroman-1-ones are a class of secondary metabolites classified under 3,4-dihydroisocoumarins, known for their diverse biological activities. Traditionally, these compounds are synthesized via halocyclization using hypervalent iodine reagents or N-bromosuccinimide (NBS), followed by hydrolysis. However, these methods often require stringent conditions and produce toxic byproducts, limiting their practicality. In this study, I employed *Curvularia inaequalis* vanadium chloroperoxidase (CVCPO) to catalyze the chemoenzymatic cyclization of 2-vinylbenzoic acids bearing various electron-donating groups (**1a–1e**). By utilizing KBr and H₂O₂ in a citrate buffer (pH 5), I achieved high yields of 4-hydroxyisochroman-1-ones (**3a–3e**). To enhance substrate solubility without compromising enzyme activity, 30% DMSO was added to the reaction mixture. This approach not only simplifies the synthesis process but also offers a more environmentally friendly alternative to conventional chemical methods. Consequently, this method provides a sustainable and efficient strategy for the synthesis of 4-hydroxyisochroman-1-ones.

School of Chemistry
Academic Year 2022

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LIST OF ABBREVIATIONS

^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
CDCl_3	Deuterated chloroform
CH_2Cl_2	Dichloromethane
CVCPO	Vanadium-dependent chloroperoxidase from <i>Curvularia inaequalis</i>
$C_p\text{VBPO}$	Vanadium bromoperoxidase from <i>Corallina pilulifera</i>
Cs_2CO_3	Cesium carbonate
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
FADH_2	Flavin adenine dinucleotide
FTIR	Fourier Transform Infrared Spectroscopy
^1H NMR	Proton Nuclear Magnetic Resonance
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HFIP	Hexafluoroisopropanol
HOX	Hypohalous acid
HRMS	High-resolution mass spectrometry
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
KBr	Potassium bromide
k_{cat}	Turnover rate
LB	Luria-Bertani
MCD	Monochlorodimedone
MeOH	Methanol
Na_2SO_4	Sodium sulfate
Na_3VO_4	Sodium orthovanadate
NaOH	Sodium hydroxide
NBS	N-Bromosuccinimide
NCS	N-Chlorosuccinimide

LIST OF ABBREVIATIONS (Continued)

Ni-NTA	Nickel-nitrilotriacetic acid
NMR	Nuclear Magnetic Resonance
OD600	Optical density at 600 nm
Oxone	Potassium peroxymonosulfate
Pd	Palladium
Pd(OAc) ₂	Palladium(II) acetate
PMSF	Phenylmethylsulfonyl fluoride
PPh ₃	Triphenylphosphine sulfide
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV-Vis	Ultraviolet-visible spectroscopy
VBrPO	Vanadium bromoperoxidase
VClPO	Vanadium chloroperoxidase
°C	Degrees Celsius
kDa	Kilodalton

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Background and significance

3,4-Dihydroisocoumarin is a crucial structural framework found in a variety of secondary metabolites with significant biological and pharmacological importance. This core structure serves as a precursor for synthesizing a range of bioactive natural products. 4-Hydroxyisochroman-1-one is a member of the polyketide family, predominantly isolated from bacterial and fungal sources (Figure 1.1) (Braca et al., 2012). Polyketides are known for their broad biological activities, including antimicrobial, anticancer, and anti-inflammatory effects (Hussain et al., 2001). The presence of 4-hydroxyisochroman-1-one and related compounds in microbial metabolites highlights their role in natural defense mechanisms and their potential as lead structures for drug discovery. Studies have shown that 4-hydroxyisochroman-1-one derivatives possess considerable biological potential, positioning them as promising candidates for drug development. These compounds exhibit cytotoxicity, suggesting applications in cancer therapy (Jansen et al., 2002; Suzuki et al., 2005; Taniguchi et al., 1996). Additionally, their strong antifungal properties make them valuable in the fight against fungal infections, particularly those caused by drug-resistant strains. Furthermore, their antibacterial effects offer potential solutions to the rising issue of antimicrobial resistance (Isaka et al., 2009). The diverse biological activities of 4-hydroxyisochroman-1-one derivatives underscore their considerable therapeutic potential, making them a key area of ongoing research. The synthesis of these compounds primarily relies on two approaches: the hetero-Diels–Alder cycloaddition of ortho-quinone dimethides followed by oxidation (Kurosaki et al., 2005), and the oxidative rearrangement of isobenzofurans followed by reduction. Both methods offer high efficiency and structural versatility, facilitating the creation of a wide range of derivatives with promising biological activities (Egan et al., 2011; Hobson et al., 2008).

However, challenges remain in the practical implementation of these methods, particularly due to limitations in substrate availability or the need for specific reaction conditions. Researchers continue to refine these synthetic strategies to improve efficiency, sustainability, and scalability for pharmaceutical and industrial applications.

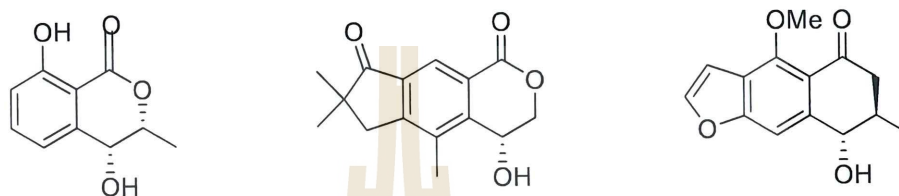


Figure 1.1 Natural products containing the 4-hydroxyisochroman-1-one skeleton.

Given the lactone structure of 4-hydroxyisochroman-1-one, it was proposed that intramolecular cyclization of 2-vinylbenzoic acid (**1a**) would be a crucial step for lactonization, facilitating the formation of the desired compound (Scheme 1.1). Therefore, the critical step involves the activation of the alkene through electrophilic halogenation, followed by the nucleophilic addition of the carboxylic acid via the 6-endo pathway. This approach favors the formation of **2**, rather than the 5-exo pathway leading to phthalide **3** (Denmark and Burk, 2010).

A novel approach utilized a catalytic amount of chiral hypervalent iodine (III), generated through the oxidation of chiral iodoarene, to facilitate the halocyclization of alkenyl benzoates. This method enabled the enantioselective synthesis of acetylated 4-hydroxyisochroman-1-one derivatives (Shimogaki et al., 2013). Subsequent hydrolysis using K_2CO_3 yielded 4-hydroxyisochroman-1-ones as the final product. In another strategy, stoichiometric *N*-bromosuccinimide (NBS) was employed to catalyze the bromocyclization of styrene-type carboxylic acids, leading to the formation of 4-bromo-3,4-dihydroisocoumarins (**2**) (Chen et al., 2012). These intermediates could then be converted into 4-hydroxyisochroman-1-one (**3a**) through a displacement reaction. However, this process had several limitations. The cyclization required multiple days to complete and a stoichiometric amount of NBS, resulting in the production of succinimide as waste. Moreover, both techniques required low temperatures and anaerobic environment to proceed efficiently. Although the 6-endo pathway was favored in both approaches, a significant amount of 5-exo cyclization occurred, generating side products.

Another reported method involved cyclization using *m*-chloroperoxybenzoic acid (*m*CPBA) and boron trifluoride (BF₃), a strong Lewis acid. However, the use of these reagents posed challenges due to their high reactivity, hazardous nature, and environmental impact, limiting the sustainability of the approach. Efforts to overcome these drawbacks have driven the search for more efficient, environmentally friendly, and scalable synthetic strategies.

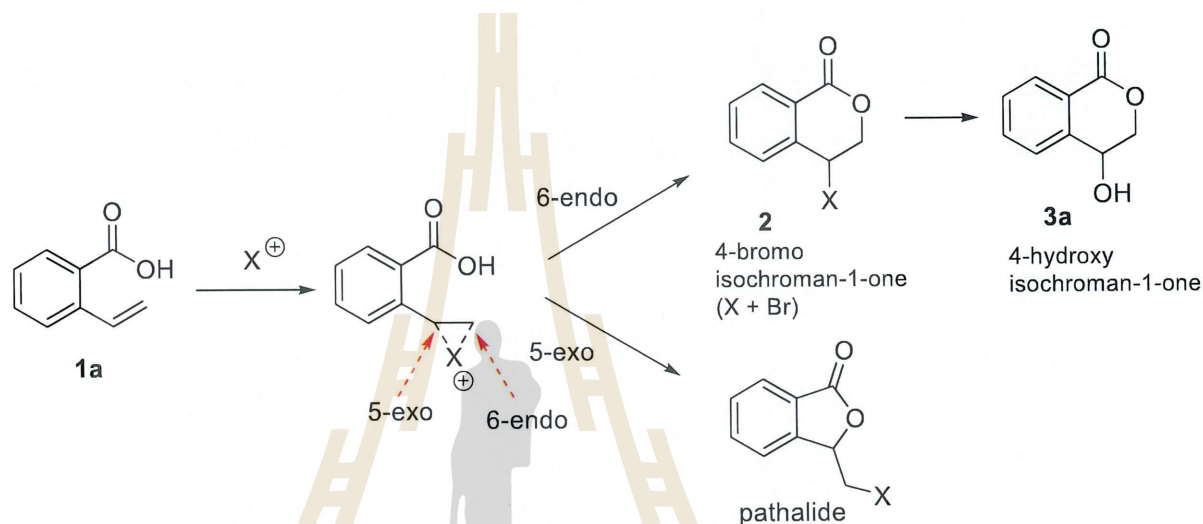


Figure 1.2 Proposed 6-endo and 5-exo halocyclization of 2-vinylbenzoic acid **1a**. The 6-endo product **2** can be finally converted to 4-hydroxyisochroman-1-one **3a**.

Historically, halogenations were carried out using hazardous and corrosive molecular halogens (X_2) (Saikia et al., 2016). Over time, organic halogenating agents like *N*-halosuccinimides (NBS, NCS) have largely replaced elemental halogens. However, the synthesis of these reagents still relies on the use of elemental dihalogens, raising environmental concerns. A more sustainable alternative involves the in-situ generation of hypohalites using mild oxidants, such as hydrogen peroxide (H₂O₂), in the presence of biocatalysts (Burek et al., 2019). This method avoids the need for elemental halogens and aligns with green chemistry principles. Notable biocatalysts include heme-dependent chloroperoxidase from *Caldariomyces fumago* (CfCVPO) (Kurosaki et al., 2005; Naapuri et al., 2017) and vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (CivCPO) (Hoeftler et al., 2020; Naapuri et al., 2022). These enzymes facilitate the oxidation of halides to hypohalites, which are then used to catalyze the halocyclization of allenols or γ,δ -unsaturated carboxylic acids and alcohols. Despite its effectiveness, CfCVPO has a significant drawback — its porphyrin

cofactor is susceptible to oxidative degradation at elevated H_2O_2 concentrations, leading to reduced enzyme stability. In contrast, *CVCPO* exhibits superior resistance to oxidative stress, maintaining catalytic activity even in the presence of high H_2O_2 concentrations and organic solvents (Hollmann, 2020). This robustness has established *CVCPO* as a preferred choice for sustainable halogenation reactions, supporting the development of greener and more efficient synthetic methodologies.

To better understand the catalytic role of *CVCPO* in the halocyclization of 2-vinylbenzoic acid (**1a**, Figure 1.2), a model reaction was conducted using KBr and H_2O_2 (Scheme 1.2). Interestingly, 4-hydroxyisochroman-1-one (**3a**) was obtained as the primary product, rather than 4-bromoisochroman-1-one (**2**), which contrasts with the outcomes typically observed in chemical halocyclization methods (Chen et al., 2012; Denmark and Burk, 2010; Shimogaki et al., 2013). Notably, the 5-exo cyclization product was not detected. However, limited solubility of 2-vinylbenzoic acid derivatives in the buffer presented a challenge. To address this, various solvent systems were tested, with a buffer containing 30% DMSO identified as the optimal cosolvent for enhancing solubility and maximizing reaction yield. Subsequent investigations revealed that only derivatives with electron-donating groups on the benzene ring successfully underwent transformation into the corresponding 4-hydroxyisochroman-1-one products. This finding highlights the influence of electronic effects on the reactivity of 2-vinylbenzoic acid derivatives.

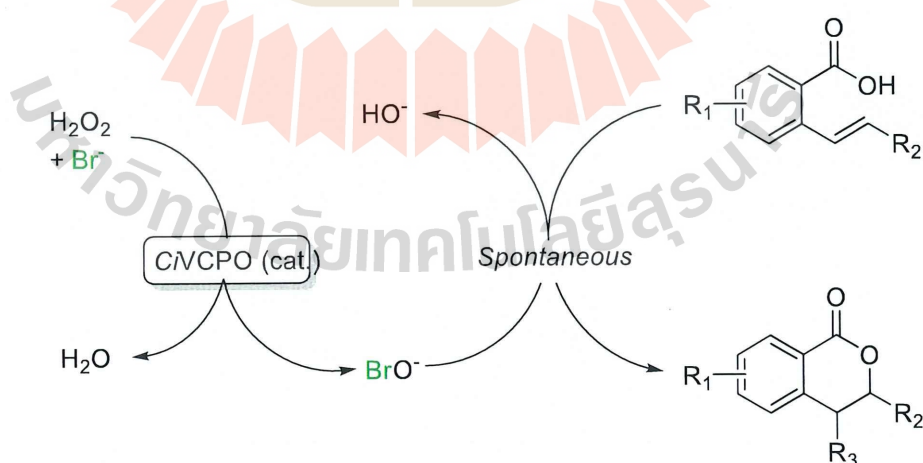


Figure 1.3 Proposed enzymatic halocyclization of 2-vinylbenzoic acid.

1.2 Research objectives

- 1) To develop the cyclization reaction of 2-vinylbenzoic acid (**1a**) catalyzed by CVCPO.
- 2) To assess the cyclization catalyzed by CVCPO using derivatives of 2-vinylbenzoic acid (**1b-h**).

1.3 Scope and limitations

- 1) The catalytic activities of CVCPO wild-type and mutant will be evaluated using a monochlorodimedone (MCD) assay to identify the more effective variant.
- 2) The selected CVCPO variant will generate hypohalite in situ to catalyze the *O*-attack cyclization of 2-vinylbenzoic acid (**1a**) in the optimized condition to maximize reaction yield.
- 3) 2-Vinylbenzoic acid derivatives (**1b-1h**) with various substituents on the benzene ring were successfully synthesized.
- 4) The optimized CVCPO reaction conditions will be used to the cyclization of synthesized 2-vinylbenzoic acid derivatives (**1b-1h**).

1.4 References

- Braca, A., Bader, A., and De Tommasi, N. (2012). Plant and fungi 3, 4-dihydroisocoumarins: Structures, biological activity, and taxonomic relationships. *Studies in Natural Products Chemistry*, 37, 191-215.
- Burek, B., Bormann, S., Hollmann, F., Bloh, J., and Holtmann, D. (2019). Hydrogen peroxide driven biocatalysis. *Green Chemistry*, 21(12), 3232-3249.
- Chen, J., Zhou, L., Tan, C. K., and Yeung, Y.-Y. (2012). An enantioselective approach toward 3, 4-dihydroisocoumarin through the bromocyclization of styrene-type carboxylic acids. *The Journal of Organic Chemistry*, 77(2), 999-1009.
- Denmark, S. E., and Burk, M. T. (2010). Lewis base catalysis of bromo- and iodolactonization, and cycloetherification. *Proceedings of the National Academy of Sciences*, 107(48), 20655-20660.
- Egan, B. A., Paradowski, M., Thomas, L. H., and Marquez, R. (2011). Regiocontrolled rearrangement of isobenzofurans. *Organic Letters*, 13(8), 2086-2089.
- Hobson, S. J., Parkin, A., and Marquez, R. (2008). Oxidative rearrangements of isobenzofurans: studies toward the synthesis of the ajudazols. *Organic Letters*, 10(13), 2813-2816.

- Hoefler, S. F., Zettl, R., Knez, D., Haberfehlner, G., Hofer, F., Rath, T., Trimmel, G., Wilkening, H. M. R., and Hanzu, I. (2020). New solar cell–battery hybrid energy system: integrating organic photovoltaics with Li-Ion and Na-Ion technologies. *ACS Sustainable Chemistry & Engineering*, 8(51), 19155-19168.
- Hollmann, R. W. R. R. F. (2020). Vanadium Catalysis. In., *The Royal Society Of Chemistry*, 548-563.
- Hussain, M. T., Rama, N. H., and Malik, A. (2001). Synthesis of some new 3-(bromophenyl) isocoumarins and their conversion to (dl)-3, 4-dihydroisocoumarins.
- Isaka, M., Yangchum, A., Intamas, S., Kocharin, K., Jones, E. G., Kongsaree, P., and Prabpai, S. (2009). Aigialomycins and related polyketide metabolites from the mangrove fungus *Aigialus parvus* BCC 5311. *Tetrahedron*. 65(22), 4396-4403.
- Jansen, R., Kunze, B., Reichenbach, H., and Höfle, G. (2002). The ajudazols A and B, novel isochromanones from *Chondromyces crocatus* (Myxobacteria): Isolation and structure elucidation. *European Journal of Organic Chemistry*. 2002(5), 917-921.
- Kurosaki, Y., Fukuda, T., and Iwao, M. (2005). Asymmetric synthesis of 3-substituted 3, 4-dihydroisocoumarins via stereoselective addition of laterally lithiated chiral 2-(o-tolyl) oxazolines to aldehydes followed by diastereomer-selective lactonization. *Tetrahedron*. 61(13), 3289-3303.
- Naapuri, J., Rolfes, J. D., Keil, J., Sapu, C. M., and Deska, J. (2017). Enzymatic halocyclization of allenic alcohols and carboxylates: a biocatalytic entry to functionalized O-heterocycles. *Green Chemistry*. 19(2), 447-452.
- Naapuri, J. M., Wagner, P. K., Hollmann, F., and Deska, J. (2022). Enzymatic Bromocyclization of α - and γ -Allenols by Chloroperoxidase from *Curvularia inaequalis*. *ChemistryOpen*. 11(1), e202100236.
- Saikia, I., Borah, A. J., and Phukan, P. (2016). Use of bromine and bromo-organic compounds in organic synthesis. *Chemical reviews*. 116(12), 6837-7042.
- Shimogaki, M., Fujita, M., and Sugimura, T. (2013). Enantioselective Oxidation of Alkenylbenzoates Catalyzed by Chiral Hypervalent Iodine (III) To Yield 4-Hydroxyisochroman-1-ones. *European Journal of Organic Chemistry*. 2013(31), 7128-7138.
- Suzuki, S., Murayama, T., and Shiono, Y. (2005). Illudalane sesquiterpenoids, echinolactones A and B, from a mycelial culture of *Echinodontium japonicum*. *Phytochemistry*. 66(19), 2329-2333.

Taniguchi, M., Yanai, M., Xiao, Y. Q., Kido, T.-i., and Baba, K. (1996). Three isocoumarins from *Coriandrum sativum*. *Phytochemistry*. 42(3), 843-846.



CHAPTER II

LITERATURE REVIEW

2.1 Coumarins and 3,4-dihydroisocoumarins

Coumarins **9** are naturally occurring compounds characterized by a fused phenolactone structure. The first extraction of coumarin **9** was from *Coumarouna odorata*, commonly known as the tonka tree (Braz Filho et al., 1980). Isocoumarins **10** and 3,4-dihydroisocoumarins **11** are structural isomers of coumarin **9**. While various substituted isocoumarins are found in nature, unsubstituted isocoumarins have not been identified in natural sources. Additionally, sulfur, selenium, and tellurium analogs **12a-12c** of these compounds have been recognized for many years (Figure 2.1).

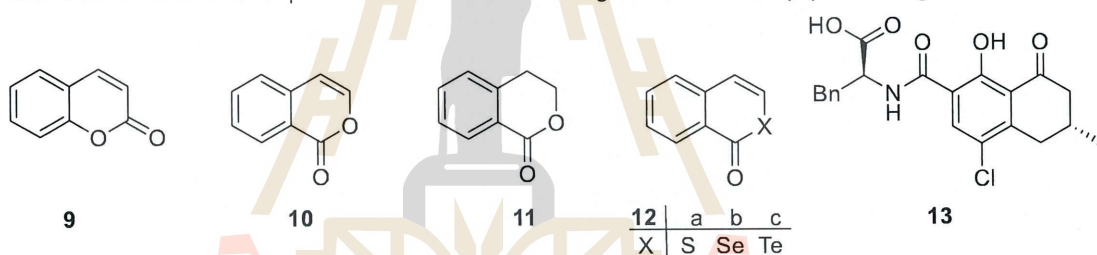


Figure 2.1 Examples of natural isocoumarins.

Isocoumarins and their related compounds are secondary metabolites generated by plants and microorganisms as a reaction to environmental factors. Certain isocoumarins are also obtained from insect pheromones and venom. These substances are crucial elements in many natural products and serve as important intermediates in the creation of various heterocyclic molecules. Isocoumarins demonstrate a variety of biological effects, including positive impacts such as antitumor, antileukemic, antiviral, and antimicrobial properties (Chan et al., 1987). Nonetheless, they can also have harmful effects. For example, ochratoxin A **13**, a mycotoxin produced by *Aspergillus* and *Penicillium* species, is recognized for its damaging effects on the liver, kidneys, and its potential to cause birth defects and cancer in animals (Zepnik et al., 2001). Given the pharmacological and biochemical properties, along with the therapeutic potential of isocoumarins and 3,4-dihydroisocoumarins, significant research has been dedicated to their isolation and synthesis.

2.2 Biological importance of 3,4-dihydroisocoumarins

3,4-Dihydroisocoumarins are a class of secondary metabolites synthesized by various organisms, including plants, fungi, and bacteria. They are commonly extracted from fungal genera such as *Aspergillus*, *Penicillium*, and *Ceratocystis*, as well as from plant species like *Flueggea* and *Coriandrum* (Singh et al., 2005). These compounds are of significant interest in pharmacological studies due to their diverse biological properties, which make them promising candidates for further research and development.

2.2.1 Antibacterial and Antifungal Activities

3,4-Dihydroisocoumarins exhibit notable antibacterial and antifungal properties, making them valuable in the field of antimicrobial research. For example, compounds such as PM-94128 (**14**) and Y-05460M-A (**15**) have shown strong antibacterial effects (Canedo et al., 1997; Huang et al., 2006). PM-94128, in particular, has been evaluated for its activity against several tumor cell lines, including P-388 (lymphoid leukemia), A-549 (human lung carcinoma), HT-29 (human colon carcinoma), and MEL-28 (human melanoma), demonstrating efficacy at concentrations around 50 nM (SATO et al., 1992). Additionally, monocerin (**16**) and its derivatives, derived from *Drechslera monoceras*, display remarkable antifungal activity against various fungal species. These compounds are particularly promising for addressing fungal infections caused by resistant pathogens like *Candida albicans* and *Aspergillus niger*, which pose a growing threat, especially to individuals with weakened immune systems (Hussain et al., 2003). Their potential in combating such infections highlights their importance in developing new therapeutic strategies.

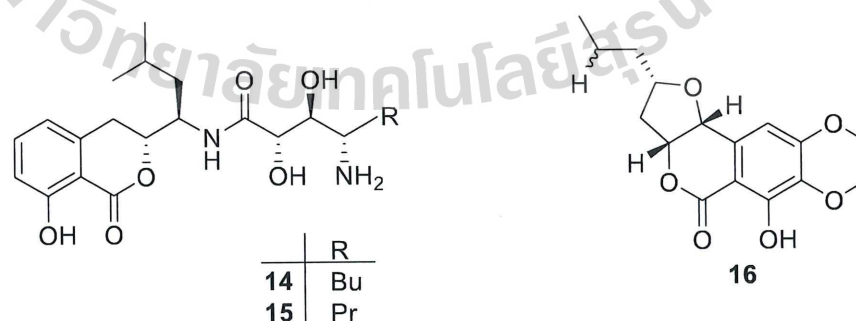


Figure 2.2 Examples of natural isocoumarins with antibacterial and antifungal activities.

2.2.2 Anticancer and Cytotoxic Activities

Several 3,4-dihydroisocoumarins have shown promising anticancer activities. Paepalantine (**17**), a 9,10-dihydroxy-5,7-dimethoxy-1H-naphtho-(2,3c)pyran-1-one, exhibits intense cytotoxic activity in the McCoy cell line. The presence and position of hydroxyl groups in the isocoumarin framework significantly influence the rate of cytotoxicity (Prithiviraj et al., 1997). Thunberginol B (**18**), another naturally occurring 3,4-dihydroisocoumarin, has been found to possess anticancer effects along with antiallergic, antimicrobial, and antioxidant activities (Devienne et al., 2002).

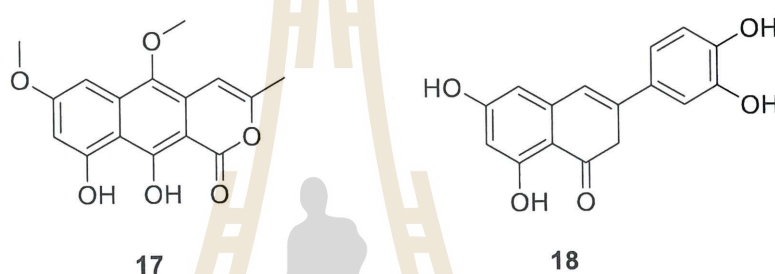


Figure 2.3 Paepalantine **17** and thunberginol B **18** have the anticancer and cytotoxic activities.

2.2.3 Anti-inflammatory and Antiulcer Activities

3,4-Dihydroisocoumarins also exhibit anti-inflammatory and antiulcer activities. Amicoumacin A (**19**), isolated from *Bacillus pumilus*, have been found to show antiulcer, antibacterial, and anti-inflammatory activities (Itoh et al., 1981). A1-77-B (**20**) is part of the A1-77s family of isocoumarins, which are antibiotics isolated from the culture broth of *Bacillus pumilus* A1-77. It exhibits a broad range of pharmacological activities, including antibacterial, anti-inflammatory, antiulcer, gastroprotective, and anti-*Helicobacter pylori* effects. It is particularly renowned for its remarkable gastroprotective activity. (Huang et al., 2006; Itoh et al., 1981).

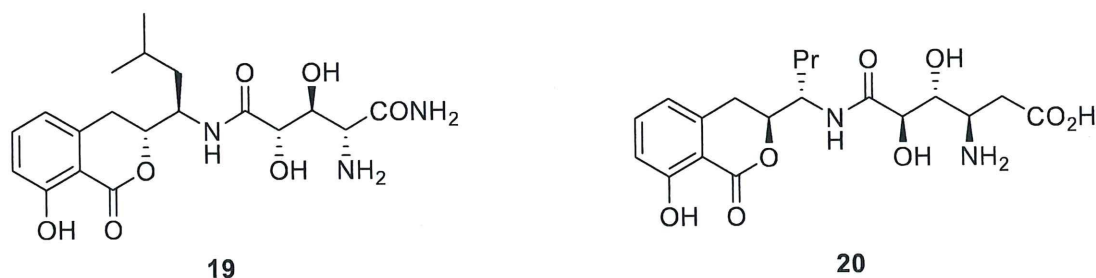


Figure 2.4 Amicoumacin A **19** and A1-77-B isocoumarin **20** have anti-inflammatory and antiulcer activities.

2.2.4 Antimalarial Activity

Malaria, a life-threatening disease caused by *Plasmodium* parasites, has been a focus of research involving 3,4-dihydroisocoumarin derivatives. Among these, mullein compounds (**21a–21d**), produced by the endophytic fungus *Botryosphaeria rhodina*, have exhibited significant antimalarial activity (Saeed, 2003). Furthermore, other derivatives such as 8-hydroxy-6-methoxy-3-pentyl-1H-isochromen-1-one, isolated from *Tessmannia densiflora*, and *halorosellins*, derived from *Halarosellinia oceanica*, have also shown promising antimalarial properties. These findings highlight the potential of 3,4-dihydroisocoumarins as a valuable resource for developing novel antimalarial therapies (Kihampa et al., 2009; Rukachaisirikul et al., 2009; Saeed, 2011).

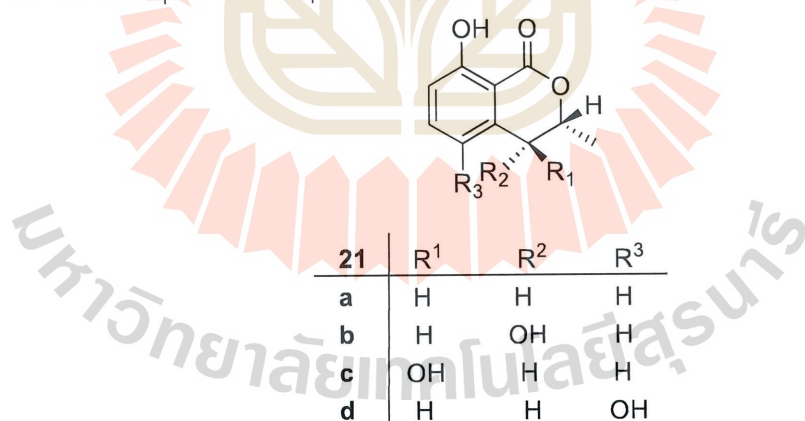


Figure 2.5 Mullein compounds have antimalarial activities.

2.3 Synthesis of 3,4-Dihydroisocoumarins

3,4-Dihydroisocoumarins are a significant class of heterocyclic compounds with diverse biological activities and applications in medicinal chemistry, natural product synthesis, and materials science. Over the years, numerous synthetic methods have been developed to access these structures.

2.3.1 Acid-Catalyzed Cyclization

One widely used approach for synthesizing 3,4-dihydroisocoumarins is acid-catalyzed cyclization. Uchiyama et al. demonstrated this strategy by selectively converting eupree carboxylic acid (**22**) into isocoumarin (**24**) through the intermediate (**23**), employing triflic acid (TfOH) or trifluoroacetic acid (CF₃COOH) as a catalyst (Figure 2.6) (Hauser and Baghdanov, 1988). This methodology was further extended to the synthesis of thunberginol A (**26**), a compound known for its antimicrobial and antiallergic properties (Figure 2.7) (Hauser and Baghdanov, 1988; Uchiyama et al., 2006; YOSHIKAWA et al., 1994; YOSHIKAWA et al., 1996).

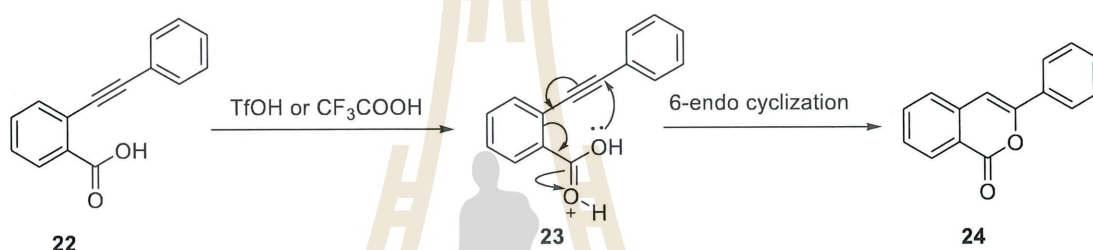


Figure 2.6 The acid-catalyzed reaction selectively transforms enecarboxylic acid **22** into isocoumarin **24**, proceeding through intermediate **23**.



Figure 2.7 The acid-catalyzed reaction converts compound **25** to thunberginol A **26** starting from compound **25**.

2.3.2 Metal-Catalyzed Cyclization

Transition metal catalysts, particularly palladium (Pd), ruthenium (Ru), and rhodium (Rh), have been widely used in the synthesis of 3,4-dihydroisocoumarins.

2.3.2.1 Ru-Catalyzed Synthesis: Menashe and colleagues demonstrated the synthesis of isocoumarin (**28**) via a ruthenium-catalyzed reaction of diphenylacetylene (**27**) with acetic acid, using Ru₃(CO)₁₂ under reflux conditions (Figure 2.8). This efficient method highlights the utility of ruthenium catalysis for selective

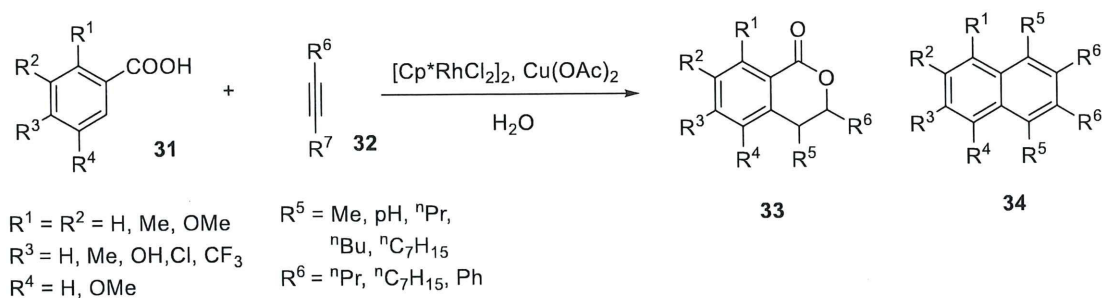


Figure 2.10 The preparation of 3,4-dihydroisocoumarin **33** by Rh-catalyzed reaction between compound **31** and **32**.

2.4 Halocyclization Approaches

2.4.1 Alkene Activation

Alkenes, distinguished by their carbon-carbon double bonds, serve as essential building blocks in organic synthesis. Efficient activation and functionalization of alkenes are pivotal for assembling complex molecular frameworks. Recent progress in alkene activation, especially via bromofunctionalization, has underscored the significant role of *N*-bromoimide reagents in enabling these transformations. (Tan and Yeung, 2013).

2.4.2 Bromofunctionalization of Alkenes

Bromofunctionalization is a widely utilized method for alkene activation, involving the addition of bromine across the carbon-carbon double bond to form a reactive bromonium ion intermediate. This intermediate is subsequently opened by nucleophiles, leading to products with high diastereoselectivity (Brown et al., 1994). The generally accepted mechanism begins with the formation of a bromonium ion when the alkene reacts with an electrophilic bromine source, such as *N*-bromoimide reagents. This three-membered bromonium intermediate is highly reactive and can be opened by nucleophiles in a stereospecific manner. The nucleophilic attack typically occurs in an anti-fashion, resulting in products with high diastereoselectivity, as illustrated in Figure 2.11. This anti-addition is attributed to the steric and electronic constraints imposed by the bromonium ion, which directs the nucleophile to attack from the opposite side of the bromine atom (Carey and Sundberg, 2007).

The selectivity and efficiency of this process make bromofunctionalization a powerful tool in organic synthesis, enabling the construction of complex molecules with precise stereochemical control. The use of *N*-bromoimide reagents, such as *N*-bromosuccinimide (NBS), further enhances the practicality of these

transformations by providing a safer and more controllable alternative to molecular bromine. This mechanistic understanding, combined with the development of chiral catalysts and Lewis acid promoters, has significantly expanded the scope of bromofunctionalization, allowing for both diastereoselective and enantioselective transformations. These advancements have opened new avenues for the synthesis of biologically active molecules and natural products, underscoring the importance of bromofunctionalization in modern synthetic chemistry (Castellanos and Fletcher, 2011; Denmark et al., 2012; Snyder, 2011; Tan and Yeung, 2013).

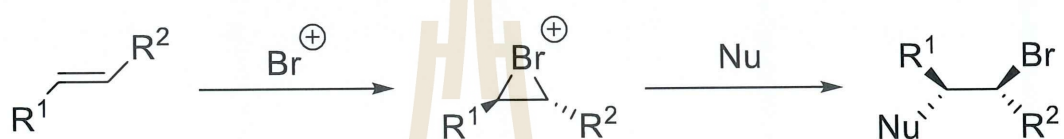


Figure 2.11 Demonstrates the core mechanism of electrophilic bromofunctionalization of alkenes.

2.4.3 Enantioselective Halocyclization

The enantioselective synthesis of 3,4-dihydroisocoumarins through bromocyclization of styrene-type carboxylic acids is a modern approach to obtain chiral dihydroisocoumarins. This method uses amino-thiocarbamate as a catalyst and *N*-bromosuccinimide (NBS) as a brominating agent, achieving high enantioselectivity and efficient production of 3-bromo-3,4-dihydroisocoumarins as the 6-endo cyclization product (Figure 2.12). The reaction involves regioselective bromine addition to the double bond of styrene-type carboxylic acids, followed by intramolecular cyclization to form the fused dihydroisocoumarin ring system (Chen et al., 2012).

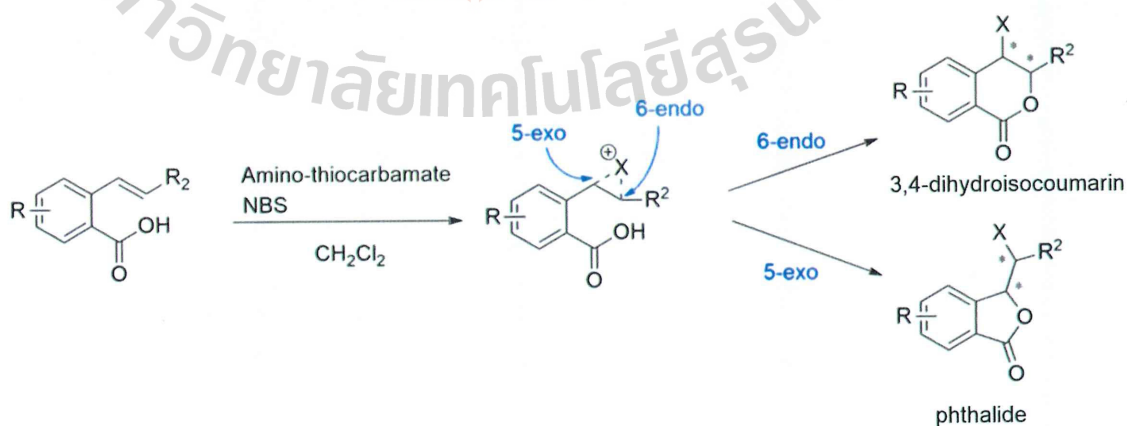


Figure 2.12 Halocyclization of 2-vinylbenzoic acid using *N*-bromosuccinimide (NBS).

Traditional (Denmark et al., 2012). In the context of halolactamization, Gandon, Leboeuf, and colleagues introduced a strategy to activate NXS while preventing deactivation of the halide source. They achieved this using hexafluoroisopropanol (HFIP) under mild conditions (Qi et al., 2021). They achieved this goal by working under gentle reaction conditions, employing hexafluoroisopropanol (HFIP) (Figure 2.13). HFIP efficiently activates NXS and prevents halide deactivation when interacting with amine functional groups. However, its reactivity is limited to arenes and oxygen nucleophiles due to their lower nucleophilicity compared to amines, which reduces the risk of side reactions involving electrophile reduction (Qi et al., 2021).

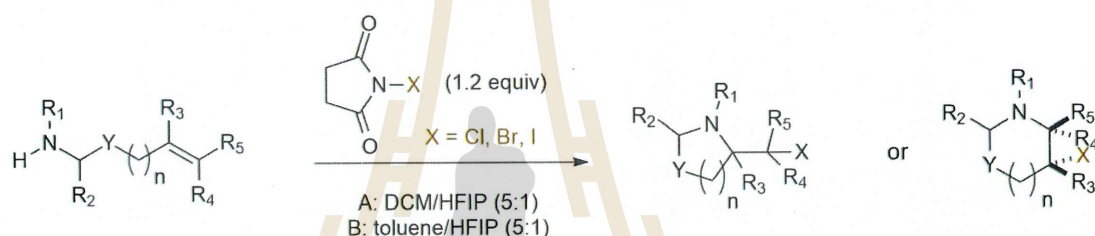


Figure 2.13 Halofunctionalization of unactivated alkenes in the presence of HFIP (Qi et al., 2021).

While current methods for producing halogenated products are feasible, they face challenges like hazardous waste, toxic reagents, and limited selectivity (Schnepel and Sewald, 2017). These issues affect functional group tolerance and substrate range. However, alternative approaches, such as biocatalysis, are gaining interest due to the unique selectivity of halogenating enzymes, which avoid the need for protecting groups or hazardous chemicals (Schnepel and Sewald, 2017).

2.5 Electrophilic Halogenation (X^+)

Electrophilic halogenation introduces halogens into electron-rich carbon centers, such as alkenes and aromatic rings. A key method for this is the use of N-bromosuccinimide (NBS), which provides controlled bromine release, enhancing selectivity and efficiency (Crowe et al., 2021; Paik et al., 2022).

Following the formation of the high-valent metal-oxo species, it interacts with a halide source resulting in the generation of an electrophilic hypohalite intermediate (Crowe et al., 2021; Paik et al., 2022; Senn, 2014). The organic substrate then reacts with this hypohalite intermediate to provide the halogenated product. Notably, the

known families of electrophilic halogenases employ various oxidants to facilitate the two-electron oxidation of X^- to X^+ (Crowe et al., 2021).

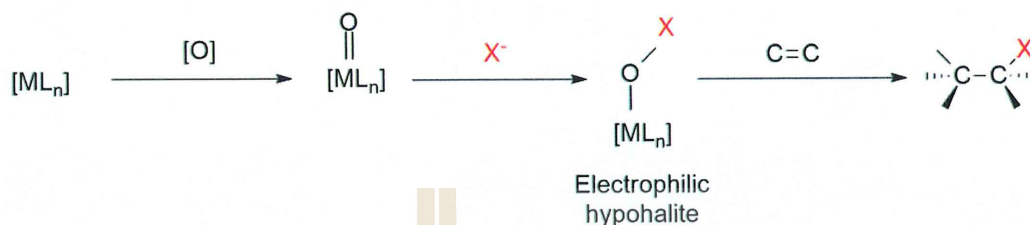


Figure 2.13 Generalized metal-catalyzed alkene halogenation.

2.6 Biocatalytic Halogenation

Biocatalysis has emerged as a promising technology for sustainable synthesis, particularly in pharmaceuticals, biotechnology, and industry. It offers several advantages over traditional methods:

- **Mild reaction conditions:** Operates at lower temperatures, reducing energy consumption.
- **Environmentally friendly solvents:** Primarily uses water, minimizing organic waste and disposal costs.
- **High selectivity:** Enzymes provide precise chemo-, regio-, and enantioselectivity, enabling efficient production of chiral compounds (Abdelraheem et al., 2019).

2.6.1 Halogenases

Halogenases, enzymes capable of activating and incorporating halogens into organic compounds, represent a critical development in biocatalysis. Initially identified as heme-dependent haloperoxidases in the 1960s, subsequent discoveries have expanded the scope of halogenase families, including vanadate-dependent haloperoxidases. These enzymes facilitate halogenation through mechanisms such as nucleophilic substitution, radical rebound, and electrophilic halide species (Senn, 2014). As researchers continue to address the limitations of traditional methods, biocatalysis offers a compelling pathway for achieving more sustainable and selective halogenation processes in the synthesis of 3,4-dihydroisocoumarins.

Halogenase families involved in electrophilic aromatic substitution include heme-dependent and vanadium-dependent haloperoxidases and flavin-dependent halogenases (Senn, 2014). The exploration of halogenation traces back to 1959 when Hager made the pioneering discovery by characterizing a fungal chlorinating enzyme as a heme-dependent chloroperoxidase (Eustáquio et al., 2008). Since then,

numerous discoveries of different classes of halogenating enzymes have revealed the utilization of three distinct redox cofactors: vanadium, the dihydro form of FAD (FADH_2), and non-heme iron centers, serving as obligatory cofactors in various types of halogenating systems. Enzymes dependent on heme and vanadium utilize hydrogen peroxide as a substrate, thereby classifying them as haloperoxidases (Senn, 2014). Indeed, the flavin-dependent halogenases rely on dioxygen as a reducible co-substrate and do not engage in reactions with hydrogen peroxide (Crowe et al., 2021; Senn, 2014). In these two categories, oxygen serves in two oxidation states: O_2 or H_2O_2 . There are notable similarities in the mechanism of activation of halogen ions for C-X bond formation by both haloperoxidases and halogenases. Haloperoxidases dependent on heme and vanadium produce bound hypohalite ($-\text{OH}$) intermediates (Crowe et al., 2021). These intermediates can function as equivalents to X^+ when reacting with electron-rich substrates. Importantly, the common theme across both categories is that the oxidation of ground state X^- ions yields the halogenating species necessary for C-X bond formation.

2.6.2 Haloperoxidases

Haloperoxidases are characterized as enzymes utilizing hydrogen peroxide and a halide to produce HOX. Haloperoxidases exhibit the least selectivity among halogenating enzymes because they catalyze the generation of free hypohalous acid, much of which is liberated by the enzyme. Heme-dependent haloperoxidases constitute a subset of haloperoxidases featuring a heme cofactor coordinated to an axial cysteine ligand within the enzyme (Figure 2.14) (Crowe et al., 2021). Hydrogen peroxide binds to the enzyme in its resting state (I), resulting in the formation of a heme-iron (IV) intermediate (II). Subsequent addition of halide to the ferryl oxygen (III) yields a heme iron (III)-OX species (IV). This process ultimately leads to the formation of free hypohalous acid (HOX), which is then utilized to react with an electrophilic substrate.

Vanadium-dependent haloperoxidases are widely found in algae, fungi, and bacteria and utilize a vanadium cofactor bound by an axial histidine ligand (Scheme 2.10b) (Crowe et al., 2021). The resting state of vanadium-dependent haloperoxidases is bound by the imidazole ring of a histidine residue (I). However, the protein ligand is used to anchor the redox cofactor in the active site. The mechanism below shows that the vanadate ion coordinates with the incoming hydrogen peroxide and gives rise to the formation of the peroxo complex (III). Following the oxidation of the halide ion, the V-OX species (IV) is generated. Subsequent hydrolysis leads to the formation of a hypohalous acid species (HOX) as the electrophilic halogenating agent.

Importantly, during the generation of the halogenating cycle, the oxidation state of the vanadium ion remains unchanged. Unlike the well-understood catalytic cycle of heme-dependent haloperoxidases, the precise sequence of events in the vanadium-dependent catalytic cycle is not as well-established, although several sources have proposed potential pathways (Crowe et al., 2021; Senn, 2014). Additional details regarding vanadium-dependent haloperoxidases will be further expounded upon in the subsequent section of the thesis. The primary distinction from the heme-iron-dependent biocatalytic cycle lies in the proposal that in the vanadium-dependent biocatalytic cycle, the vanadium center retains its oxidation state throughout the process (Senn, 2014).

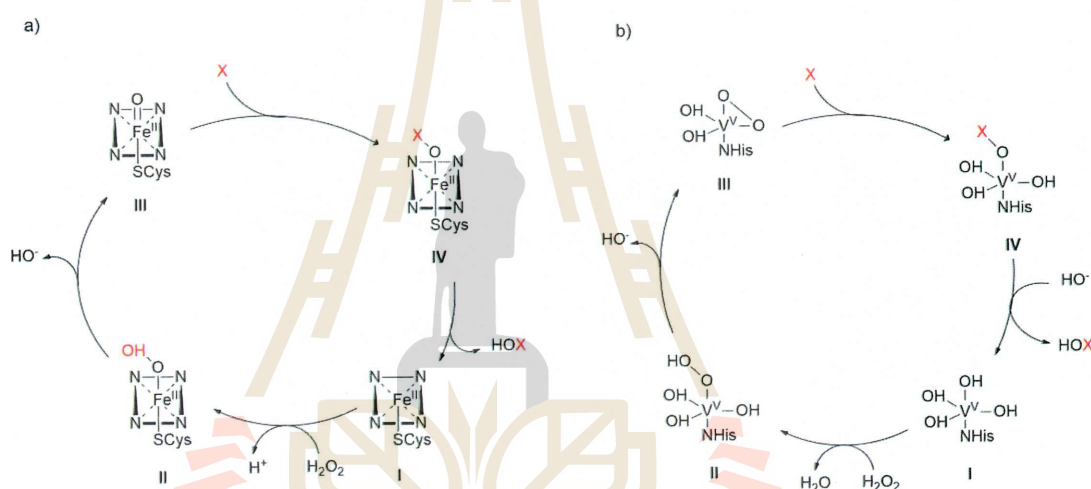


Figure 2.14 HOX generating mechanism catalyzed by (a) heme -dependent haloperoxidases and (b) vanadium-dependent haloperoxidases (Senn, 2014).

2.6.3 Vanadium dependent Haloperoxidases

As previously discussed, both vanadium haloperoxidases and heme haloperoxidases catalyze electrophilic halofunctionalization. The literatures indicate that high concentrations of hydrogen peroxide may impede the catalytic activity of haloperoxidases (Soedjak et al., 1995). Another significant distinction between the two types of reactions is that certain vanadium haloperoxidases have diverse applications. These applications encompass the degradation of lignocellulose, halocyclization, regiospecific halogenation of organic substrates, and antifouling activities (Winter and Moore, 2009).

Vanadium is the second most abundant of all the transition metal elements and is commonly found in the ocean (Butler and Carter-Franklin, 2004; Martinez et al., 2001; Michibata, 2011; Tschirret-Guth and Butler, 1994). Vanadium is also frequently

encountered in the form of a vanadate oxyanion at a neutral pH of 7 (Crans et al., 2004; Martinez et al., 2001). Additional investigations have corroborated the presence of the cofactor in terrestrial fungi and marine bacteria. Among the halides utilized in vanadium haloperoxidases, bromide is notably abundant, especially in marine ecosystems. In a significant breakthrough in 1984, Vilter and colleagues identified the first vanadium haloperoxidase in seaweed (Vilter, 1984). They discovered a competitive kinetic transformation utilizing vanadium bromoperoxidase from the brown algae, *Ascophyllum nodosum* (Vilter, 1984). This enzyme is responsible for the biosynthesis of halogenated natural products. However, it's noteworthy that additional products could be formed, as the hypobromous acid can diffuse from the enzyme's active site and halogenate substrates that are not bound to the enzyme (Vilter, 1984). Other oxidized intermediates of vanadium bromoperoxidase include bromine, tribromide, and an enzyme-bound "bromonium ion equivalent" (Butler and Walker, 1993). Interestingly, there are relatively few examples of natural systems that utilize vanadium ions. Among them, nitrogenases and haloperoxidases are prominent. However, this section will primarily focus on discussing haloperoxidases (Burns et al., 1971; Butler and Sandy, 2009; Martinez et al., 2001; Schweitzer et al., 2008). Since this initial discovery, several reports have provided support for the presence of vanadium haloperoxidases in various organisms such as seaweed, fungi, and lichens (Michibata, 2011). Only three vanadium haloperoxidases have had their crystal structure resolved: from the fungus *C. inaequalis*, VCPO, from the brown alga, *A. nodosum*, VBPO, and from the red alga *C. officinalis*, VBPO (Isupov et al., 2000; Macedo-Ribeiro et al., 1999; McLauchlan et al., 2018; Messerschmidt et al., 1997; Messerschmidt and Wever, 1996; Weyand et al., 1999).

While there is debate in the literature regarding the mechanism of vanadium haloperoxidases, sources concur that the oxidation state of vanadium remains constant throughout the catalytic cycle (Rehder, 1999). Scheme 7 illustrates the simplified proposed mechanism (Butler and Sandy, 2009; Colpas et al., 1996; Rauegi and Carloni, 2006; Winter and Moore, 2009). The initial step in catalysis involves the coordination of hydrogen peroxide to the vanadium center. A histidine residue linked to the metal center (I) activates an axial water molecule for deprotonation, facilitating peroxide binding. The crystal structure of the peroxovanadium center shows peroxide coordination in the equatorial plane, inducing distortion in the vanadium site to a tetragonal bipyramidal geometry (Macedo-Ribeiro et al., 1999). Once peroxide has bound to the site (II), histidine is no longer hydrogen-bonded to any oxygen atoms of the cofactor, allowing lysine in the active site to come into direct contact with one of

the oxygen atoms of the bound peroxide. This interaction further activates the peroxide through charge separation. Subsequently, a halide ion is introduced into the cycle and binds to the enzyme via a nucleophilic attack on the partially positive oxygen atom (III). This binding disrupts the peroxide bond, generating the nucleophilic XO^- species, which exits the coordination sphere as hypohalous acid after protonation from a water molecule. In the presence of an appropriate nucleophile, the resulting hypohalous acid intermediate reacts with the organic substrate, leading to the formation of the halogenated product, IV, as depicted in Figure 2.15. In the absence of a substrate, the oxidized halogen intermediate reacts with another equivalent of hydrogen peroxide, yielding dioxygen in the singlet state and the halide.

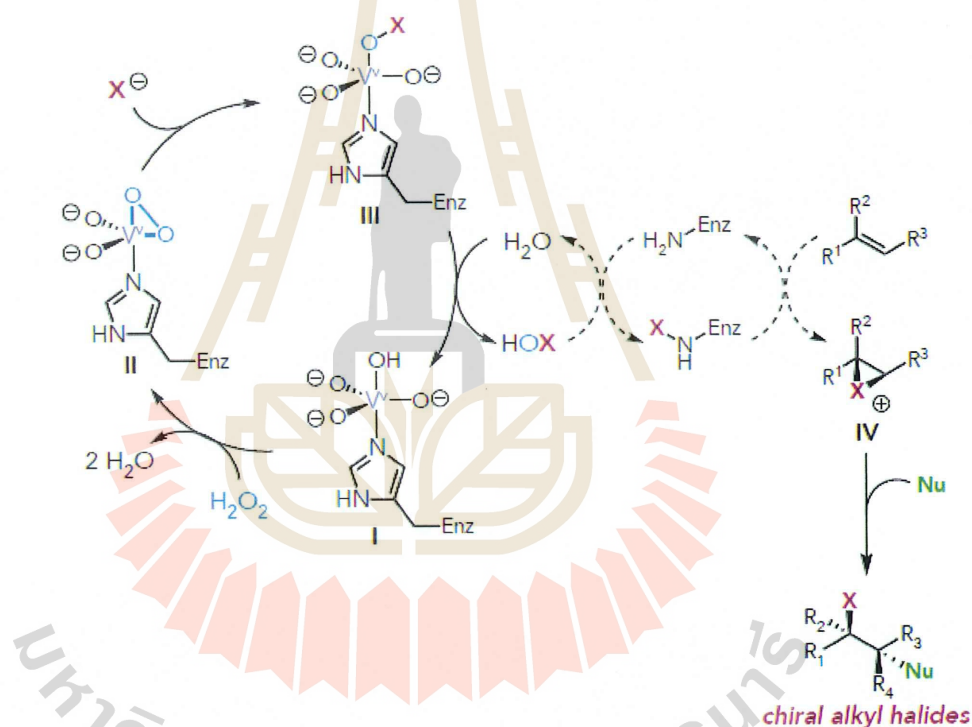


Figure 2.15 Proposed catalytic cycle of vanadium-dependent haloperoxidases generates hypohalite to undergo electrophilic addition of alkene (Butler and Sandy, 2009).

2.7 Recent studies on vanadium-dependent haloperoxidases

2.7.1 Enzyme activity studies

Enzyme engineering encompasses methodologies aimed at modifying enzyme properties to suit specific industrial requirements (Singh et al., 2013). Directed evolution techniques have been employed to enhance the activity of vanadium-

chloroperoxidase (VCPO) from the species *C. inaequalis*, particularly at mildly alkaline pH (Kaur and Sharma, 2006). Initial kinetic studies revealed a notable contrast in VCPO activity between acidic and alkaline pH environments. While the enzyme demonstrated efficient oxidation of bromide at pH 5 (with a turnover rate, k_{cat} , of approximately 100 s^{-1}), its activity dramatically decreased at a slightly alkaline pH typical of seawater (pH 8), showing a significantly lower k_{cat} value of only 1 s^{-1} . This discrepancy highlights a limitation in VCPO functionality under conditions resembling its natural habitat (Hemrika et al., 1999). In a recent study, researchers successfully engineered a triple mutant variant of VCPO (P395D/L241V/T343A), resulting in a remarkable enhancement of enzymatic activity across different pH conditions. The mutant exhibited an astounding 100-fold increase in activity at pH 8 compared to the wild-type enzyme. Additionally, it displayed notable improvements in brominating and chlorinating activities at pH 5 (Kaur and Sharma, 2006).

2.7.2 Haloperoxidases catalysts in organic synthesis

Haloperoxidases have been extensively studied for their ability to catalyze the addition of hypohalites, generated in situ, to carbon-carbon double bonds ($\text{C}=\text{C}$), resulting in the formation of halohydrins (Figure 2.16). This reaction has been a subject of interest since the 1980s, with pioneering work by Itoh et al. exploring the halohydroxylation of various alkenes using enzymes (Figure 2.17) such as *LfCPO* (from *Leptoxylum fumago*) (Itoh et al., 1987) and *CpVBPO* (from *Corallina pilulifera*) (Itoh et al., 1987; Itoh et al., 1988). These reactions were typically conducted in aqueous media, where hydroxide ions (OH^-) served as the predominant nucleophile, leading to the formation of halohydrins. The selectivity of these reactions adhered to Markovnikov's rule, consistent with the expected chemical behavior (Yamada et al., 1985). In more recent studies, Schrader and colleagues expanded the scope of halohydroxylation reactions by utilizing terpenes as substrates (Figure 2.6). This advancement demonstrated the versatility of haloperoxidases in facilitating the functionalization of complex natural products, further highlighting their potential in synthetic organic chemistry (Kaup et al., 2007; Piantini et al., 2011).

The study by Höfler and colleagues presents a chemoenzymatic approach for the bromolactonization of 4-pentenoic acid (**36**) at a preparative scale, utilizing the vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (CVCPO) to generate hypobromite *in situ* from bromide and hydrogen peroxide (Figure 2.18). This method addresses the challenges associated with the use of stoichiometric halogenating agents, such as safety concerns, waste generation, and side reactions. The authors successfully scaled up the reaction to a 10 L scale, achieving high yields of the

desired bromolactone product (**36**). They also identified substrate inhibition as a limiting factor and implemented a fed-batch strategy to mitigate this issue. The study highlights the potential of chemoenzymatic methods as a sustainable alternative to traditional chemical processes, with applications in the synthesis of functionalizable polyesters (Höfler et al., 2020).

This body of work demonstrates the broad utility of haloperoxidase-catalyzed halohydroxylation reactions, with ongoing research focused on optimizing reaction conditions for practical applications in the synthesis of halogenated compounds.

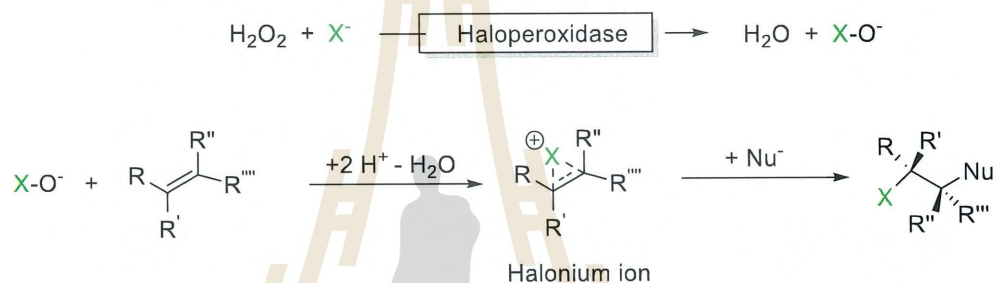


Figure 2.16 Haloperoxidase-catalyzed halohydroxylation of alkenes.

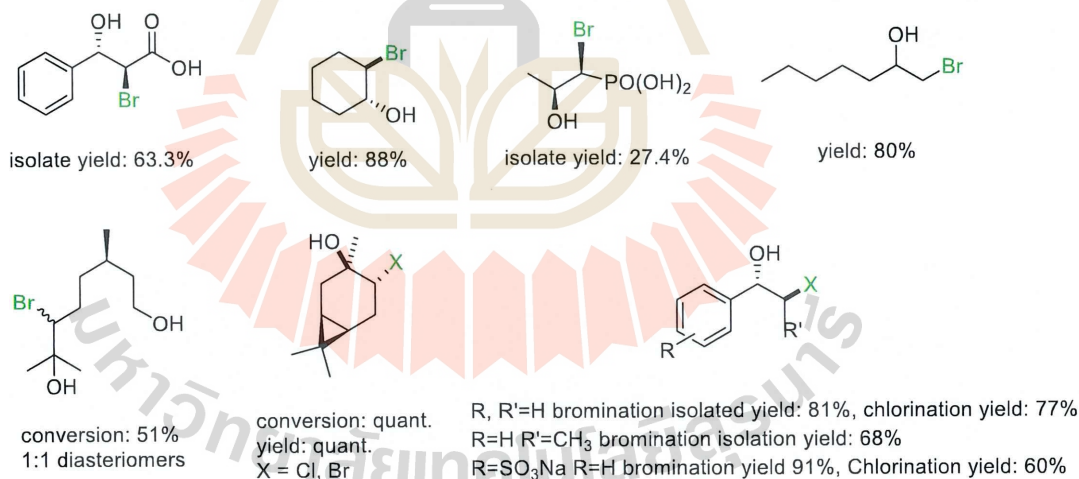


Figure 2.17 Halohydrins were synthesized through haloperoxidase-catalyzed reactions of alkenes.

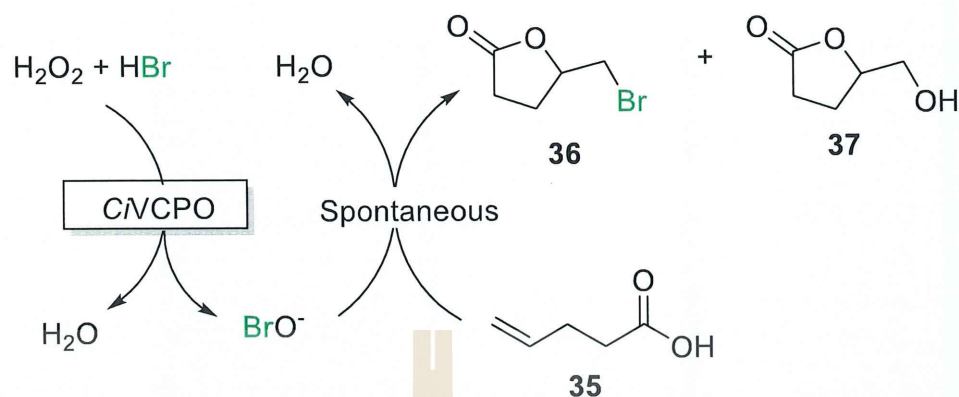


Figure 2.18 The chemoenzymatic bromolactonization of 4-pentenoic acid (**35**) resulted in the formation of 5-(bromomethyl)dihydrofuran-2(3H)-one (**36**) and the byproduct 5-(hydroxymethyl)dihydrofuran-2(3H)-one (**37**).

2.8 References

- Abdelraheem, E. M., Busch, H., Hanefeld, U., and Tonin, F. (2019). Biocatalysis explained: from pharmaceutical to bulk chemical production. *Reaction Chemistry & Engineering*. 4(11), 1878-1894.
- Braz Filho, R., De Moraes, M. P., and Gottlieb, O. R. (1980). Pterocarpanes from *Swartzia laevis*. *Phytochemistry*. 19(9), 2003-2006.
- Brown, R., Nagorski, R., Bennet, A., McClung, R., Aarts, G., Klobukowski, M., McDonald, R., and Santarsiero, B. (1994). Stable bromonium and iodonium ions of the hindered olefins adamantylideneadamantane and bicyclo [3.3. 1] nonylidenebicyclo [3.3. 1] nonane. X-ray structure, transfer of positive halogens to acceptor olefins, and ab initio studies. *Journal of the American Chemical Society*. 116(6), 2448-2456.
- Burns, R., Fuchsman, W., and Hardy, R. (1971). Nitrogenase from vanadium-grown *Azotobacter*: isolation, characteristics, and mechanistic implications. *Biochemical and biophysical research communications*. 42(3), 353-358.
- Butler, A., and Carter-Franklin, J. N. (2004). The role of vanadium bromoperoxidase in the biosynthesis of halogenated marine natural products. *Natural Product Reports*. 21(1), 180-188.
- Butler, A., and Sandy, M. (2009). Mechanistic considerations of halogenating enzymes. *Nature*. 460(7257), 848-854.
- Butler, A., and Walker, J. (1993). Marine haloperoxidases. *Chemical Reviews*. 93(5), 1937-1944.
- Canedo, L. M., PUENTES, J. L. F., BAZ, J. P., ACEBAL, C., DE LA CALLE, F., GRÁVALOS, D. G., and DE QUESADA, T. G. (1997). PM-94128, a new isocoumarin antitumor

- agent produced by a marine bacterium. *The Journal of antibiotics*. 50(2), 175-176.
- Carey, F. A., and Sundberg, R. J. (2007). Advanced organic chemistry: part A: structure and mechanisms. *Springer Science & Business Media*.
- Castellanos, A., and Fletcher, S. P. (2011). Current methods for asymmetric halogenation of olefins. *Chemistry—A European Journal*. 17(21), 5766-5776.
- Chan, W. R., Tinto, W. F., Manchand, P. S., and Todaro, L. J. (1987). Stereostructures of geodiamolides A and B, novel cyclodepsipeptides from the marine sponge *Geodia* sp. *The Journal of Organic Chemistry*. 52(14), 3091-3093.
- Chen, J., Zhou, L., Tan, C. K., and Yeung, Y.-Y. (2012). An enantioselective approach toward 3, 4-dihydroisocoumarin through the bromocyclization of styrene-type carboxylic acids. *The Journal of Organic Chemistry*. 77(2), 999-1009.
- Colpas, G. J., Hamstra, B. J., Kampf, J. W., and Pecoraro, V. L. (1996). Functional models for vanadium haloperoxidase: reactivity and mechanism of halide oxidation. *Journal of the American Chemical Society*. 118(14), 3469-3478.
- Connors, R., Tran, E., and Durst, T. (1996). Acyl cyanides as carbonyl heterodienophiles: application to the synthesis of naphthols, isoquinolones, and isocoumarins. *Canadian journal of chemistry*. 74(2), 221-226.
- Crans, D. C., Smee, J. J., Gaidamauskas, E., and Yang, L. (2004). The chemistry and biochemistry of vanadium and the biological activities exerted by vanadium compounds. *Chemical reviews*. 104(2), 849-902.
- Crowe, C., Molyneux, S., Sharma, S. V., Zhang, Y., Gkotsi, D. S., Connaris, H., and Goss, R. J. (2021). Halogenases: a palette of emerging opportunities for synthetic biology—synthetic chemistry and C–H functionalisation. *Chemical Society Reviews*. 50(17), 9443-9481.
- Denmark, S. E., Kuester, W. E., and Burk, M. T. (2012). Catalytic, asymmetric halofunctionalization of alkenes—a critical perspective. *Angewandte Chemie International Edition*. 51(44), 10938-10953.
- Devienne, K. F., Raddi, M. S. G., Varanda, E. A., and Vilegas, W. (2002). In vitro cytotoxicity of some natural and semi-synthetic isocoumarins from *Paepalanthus bromelioides*. *Zeitschrift für Naturforschung C*. 57(1-2), 85-88.
- Eustáquio, A. S., Pojer, F., Noel, J. P., and Moore, B. S. (2008). Discovery and characterization of a marine bacterial SAM-dependent chlorinase. *Nature chemical biology*. 4(1), 69-74.
- Hauser, F. M., and Baghdanov, V. M. (1988). A new procedure for regiospecific syntheses of benzopyran-1-ones. *The Journal of Organic Chemistry*. 53(20), 4676-4681.

- Hemrika, W., Renirie, R., Macedo-Ribeiro, S., Messerschmidt, A., and Wever, R. (1999). Heterologous Expression of the Vanadium-containing Chloroperoxidase from *Curvularia inaequalis* in *Saccharomyces cerevisiae* and Site-directed Mutagenesis of the Active Site Residues His496, Lys353, Arg360, and Arg490. *Journal of biological chemistry*. 274(34), 23820-23827.
- Höfler, G. T., But, A., Younes, S. H., Wever, R., Paul, C. E., Arends, I. W., and Hollmann, F. (2020). Chemoenzymatic halocyclization of 4-pentenoic acid at preparative scale. *ACS sustainable chemistry & engineering*. 8(7), 2602-2607.
- Huang, Y.-F., Li, L.-H., Tian, L., Qiao, L., Hua, H.-M., and Pei, Y.-H. (2006). Sg17-1-4, a novel isocoumarin from a marine fungus *Alternaria tenuis* Sg17-1. *The Journal of Antibiotics*. 59(6) 355-357.
- Hussain, M., Hussain, M. T., Rama, N. H., Hameed, S., Malik, A., and Khan, K. M. (2003). Synthesis and antimicrobial activities of some isocoumarin and dihydroisocoumarin derivatives. *Natural Product Research*. 17(3), 207-214.
- Isupov, M. N., Dalby, A. R., Brindley, A. A., Izumi, Y., Tanabe, T., Murshudov, G. N., and Littlechild, J. A. (2000). Crystal structure of dodecameric vanadium-dependent bromoperoxidase from the red algae *Corallina officinalis*. *Journal of molecular biology*. 299(4), 1035-1049.
- Itoh, J., OMOTO, S., SHOMURA, T., NISHIZAWA, N., MIYADO, S., YUDA, Y., SHIBATA, U., and INOUE, S. (1981). Amicoumacin-A, a new antibiotic with strong antiinflammatory and antiulcer activity. *The Journal of antibiotics*. 34(5), 611-613.
- Itoh, N., Cheng, L. Y., Izumi, Y., and Yamada, H. (1987). Immobilized bromoperoxidase of *Corallina pilulifera* as a multifunctional halogenating biocatalyst. *Journal of Biotechnology*. 5(1), 29-38.
- Itoh, N., Hasan, A. Q., Izumi, Y., and Yamada, H. (1988). Substrate specificity, regiospecificity and stereospecificity of halogenation reactions catalyzed by non-heme-type bromoperoxidase of *Corallina pilulifera*. *European journal of biochemistry*. 172(2), 477-484.
- Kaup, B.-A., Piantini, U., Wüst, M., and Schrader, J. (2007). Monoterpenes as novel substrates for oxidation and halo-hydroxylation with chloroperoxidase from *Caldariomyces fumago*. *Applied microbiology and biotechnology*. 73, 1087-1096.
- Kaur, J., and Sharma, R. (2006). Directed evolution: an approach to engineer enzymes. *Critical reviews in biotechnology*. 26(3), 165-199.

- Kihampa, C., Nkunya, M. H., Joseph, C. C., Magesa, S. M., Hassanali, A., Heydenreich, M., and Kleinpeter, E. (2009). Anti-mosquito and antimicrobial nor-halimanoids, isocoumarins and an anilinoide from *Tessmannia densiflora*. *Phytochemistry*. 70(10), 1233-1238.
- Macedo-Ribeiro, S., Hemrika, W., Renirie, R., Wever, R., and Messerschmidt, A. (1999). X-ray crystal structures of active site mutants of the vanadium-containing chloroperoxidase from the fungus *Curvularia inaequalis*. *JBIC Journal of Biological Inorganic Chemistry*. 4(2), 209-219.
- Marchal, E., Uriac, P., Legouin, B., Toupet, L., and van de Weghe, P. (2007). Cycloisomerization of γ - and δ -acetylenic acids catalyzed by gold (I) chloride. *Tetrahedron*. 63(40), 9979-9990.
- Martinez, J. S., Carroll, G. L., Tschirret-Guth, R. A., Altenhoff, G., Little, R. D., and Butler, A. (2001). On the regiospecificity of vanadium bromoperoxidase. *Journal of the American Chemical Society*. 123(14), 3289-3294.
- McLauchlan, C. C., Murakami, H. A., Wallace, C. A., and Crans, D. C. (2018). Coordination environment changes of the vanadium in vanadium-dependent haloperoxidase enzymes. *Journal of Inorganic Biochemistry*. 186, 267-279.
- Messerschmidt, A., Prade, L., and Wever, R. (1997). Implications for the catalytic mechanism of the vanadium-containing enzyme chloroperoxidase from the fungus *Curvularia inaequalis* by X-ray structures of the native and peroxide form.
- Messerschmidt, A., and Wever, R. (1996). X-ray structure of a vanadium-containing enzyme: chloroperoxidase from the fungus *Curvularia inaequalis*. *Proceedings of the National Academy of Sciences*. 93(1), 392-396.
- Michibata, H. (2011). Vanadium: biochemical and molecular biological approaches. *Springer Science & Business Media*.
- Paik, A., Paul, S., Bhowmik, S., Das, R., Naveen, T., and Rana, S. (2022). Recent Advances in First-Row Transition-Metal-Mediated C–H Halogenation of (Hetero) arenes and Alkanes. *Asian Journal of Organic Chemistry*. 11(5), e202200060.
- Piantini, U., Schrader, J., Wawrzun, A., and Wüst, M. (2011). A biocatalytic route towards rose oxide using chloroperoxidase. *Food chemistry*. 129(3), 1025-1029.
- Prithiviraj, B., Singh, U., Manickam, M., Srivastava, J., and Ray, A. (1997). Antifungal activity of bergenin, a constituent of *Flueggea microcarpa*. *Plant Pathology*. 46(2), 224-228.

- Qi, C., Force, G., Gandon, V., and Lebœuf, D. (2021). Hexafluoroisopropanol-Promoted Haloamidation and Halolactonization of Unactivated Alkenes. *Angewandte Chemie International Edition*. 60(2), 946-953.
- Raugei, S., and Carloni, P. (2006). Structure and function of vanadium haloperoxidases. *The Journal of Physical Chemistry B*. 110(8), 3747-3758.
- Rehder, D. (1999). The coordination chemistry of vanadium as related to its biological functions. *Coordination Chemistry Reviews*. 182(1), 297-322.
- Rukachaisirikul, V., Arunpanichlert, J., Sukpondma, Y., Phongpaichit, S., and Sakayaroj, J. (2009). Metabolites from the endophytic fungi *Botryosphaeria rhodina* PSU-M35 and PSU-M114. *Tetrahedron*. 65(51), 10590-10595.
- Saeed, A. (2003). Synthesis of 8-Desoxythunberginol A and (±)-8-Desoxy-3, 4-Dihydrothunberginol A. *Journal of the Chinese Chemical Society*. 50(2), 313-317.
- Saeed, A. (2011). Synthesis of 8-hydroxy-6-methoxy-3-pentyl-1H-isochromen-1-one from *Tessmannia densiflora*. *European Journal of Chemistry*. 2(1), 117-119.
- SATO, T., NAGAI, K., SUZUKI, K., MORIOKA, M., SAITO, T., NOHARA, C., SUSAKI, K., and TAKEBAYASHI, Y. (1992). A new isocoumarin antibiotic, Y-05460M-A. *The Journal of Antibiotics*. 45(12), 1949-1952.
- Schnepel, C., and Sewald, N. (2017). Enzymatic halogenation: a timely strategy for regioselective C–H activation. *Chemistry–A European Journal*. 23(50), 12064-12086.
- Schweitzer, A., Gutmann, T., Wächter, M., Breitzke, H., Buchholz, A., Plass, W., and Buntkowsky, G. (2008). 51V solid-state NMR investigations and DFT studies of model compounds for vanadium haloperoxidases. *Solid State Nuclear Magnetic Resonance*. 34(1-2), 52-67.
- Senn, H. M. (2014). Insights into enzymatic halogenation from computational studies. *Frontiers in Chemistry*. 2, 98.
- Singh, I. P., Bharate, S. B., and Bhutani, K. (2005). Anti-HIV natural products. *Current science*, 269-290.
- Singh, R. K., Tiwari, M. K., Singh, R., and Lee, J.-K. (2013). From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes. *International journal of molecular sciences*. 14(1), 1232-1277.
- Snyder, S. A. (2011). Halonium-induced cyclization reactions. *Aldrichimica Acta*. 44: 27.
- Soedjak, H. S., Walker, J., and Butler, A. (1995). Inhibition and inactivation of vanadium bromoperoxidase by the substrate hydrogen peroxide and further mechanistic studies. *Biochemistry*. 34(39), 12689-12696.

- Tan, C. K., and Yeung, Y.-Y. (2013). Recent advances in stereoselective bromofunctionalization of alkenes using N-bromoamide reagents. *Chemical Communications*. 49(73), 7985-7996.
- Tschirret-Guth, R. A., and Butler, A. (1994). Evidence for organic substrate binding to vanadium bromoperoxidase. *Journal of the American Chemical Society*. 116(1), 411-412.
- Uchiyama, M., Ozawa, H., Takuma, K., Matsumoto, Y., Yonehara, M., Hiroya, K., and Sakamoto, T. (2006). Regiocontrolled Intramolecular Cyclizations of Carboxylic Acids to Carbon–Carbon Triple Bonds Promoted by Acid or Base Catalyst. *Organic Letters*. 8(24), 5517-5520.
- Vilter, H. (1984). Peroxidases from Phaeophyceae: a vanadium (V)-dependent peroxidase from *Ascophyllum nodosum*. *Phytochemistry*. 23(7), 1387-1390.
- Weyand, M., Hecht, H.-J., Kiess, M., Liaud, M.-F., Vilter, H., and Schomburg, D. (1999). X-ray structure determination of a vanadium-dependent haloperoxidase from *Ascophyllum nodosum* at 2.0 Å resolution. *Journal of molecular biology*. 293(3), 595-611.
- Winter, J. M., and Moore, B. S. (2009). Exploring the chemistry and biology of vanadium-dependent haloperoxidases. *Journal of Biological Chemistry*. 284(28), 18577-18581.
- Yamada, H., Itoh, N., and Izumi, Y. (1985). Chloroperoxidase-catalyzed halogenation of trans-cinnamic acid and its derivatives. *Journal of Biological Chemistry*. 260(22), 11962-11969.
- YOSHIKAWA, M., HARADA, E., NAITOH, Y., INOUE, K., MATSUDA, H., SHIMODA, H., YAMAHARA, J., and MURAKAMI, N. (1994). Development of bioactive functions in *Hydrangeae Dulcis Folium*. III. On the antiallergic and antimicrobial principles of *Hydrangeae Dulcis Folium*.(1). Thunberginols A, B, and F. *Chemical and pharmaceutical bulletin*. 42(11), 2225-2230.
- YOSHIKAWA, M., MATSUDA, H., SHIMODA, H., SHIMADA, H., HARADA, E., NAITOH, Y., MIKI, A., YAMAHARA, J., and MURAKAMI, N. (1996). Development of bioactive functions in *hydrangeae dulcis folium*. V. On the antiallergic and antimicrobial principles of *hydrangeae dulcis folium*.(2). Thunberginols C, D, and E, thunberginol G 3'-O-glucoside,(-)-hydrangenol 4'-o-glucoside, and (+)-hydrangenol 4'-O-glucoside. *Chemical and pharmaceutical bulletin*. 44(8), 1440-1447.
- YOSHIKAWA, M., UCHIDA, E., CHATANI, N., MURAKAMI, N., and YAMAHARA, J. (1992). Thunberginols A, B, and F, new antiallergic and antimicrobial principles from

hydrangeae dulcis folium. *Chemical and pharmaceutical bulletin*. 40(11), 3121-3123.

Zepnik, H., Pähler, A., Schauer, U., and Dekant, W. (2001). Ochratoxin A-induced tumor formation: is there a role of reactive ochratoxin A metabolites. *Toxicological Sciences*. 59(1), 59-67.



CHAPTER III

MATERIALS AND METHODS

3.1 General experimental information

3.1.1 Materials and Instruments

Laboratory materials and preparation will take place in Asst. Dr. Rung-Yi Lai's lab and the Center of Science and Technological Equipment at Suranaree University of Technology. All reagents were purchased from commercial sources (Sigma Aldrich, TCI, Carlo Erba, Acros, and Merck) and used without further purification unless otherwise specified. All reactions were performed with magnetic stirring unless noted otherwise. Absorption spectra will be recorded using a UV-Vis spectrophotometer (Agilent Technologies Cary 300).

3.1.2 Chromatography

Thin layer chromatography (TLC) was analyzed under UV light. Column chromatography was performed using silica gel (300-400 mesh). Organic solvents were removed under reduced pressure using a rotary evaporator.

3.1.3 Product characterization

^1H NMR and ^{13}C NMR spectra were recorded on a 500 MHz (^1H) and 125 MHz (^{13}C) NMR spectrometer (Avance, Spectrometer, Bruker Biospin Ag, USA) at room temperature in CDCl_3 . Trans-cinnamic acid was used as an internal standard to determine the conversions or yields of CVCPO-catalyzed reactions. Chemical shifts of ^1H NMR spectra were recorded and reported in ppm from the solvent resonance (CDCl_3 at 7.26 ppm). Data were reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, br = broad, and m = multiplet), coupling constant in Hertz (Hz) and integration. ^{13}C NMR spectra were also recorded in ppm from the solvent resonance (CDCl_3 at 77.0 ppm). High-resolution mass spectrometry was performed on a Bruker Microflex MALDI-TOF. UV-Vis-NIR spectrophotometer (Shimadzu/UV-1900i) was used to conduct enzyme kinetic experiments. Fourier transform infrared spectrophotometer (FTIR, PerkinElmer Spectrum GX) was used to collect IR spectra, whose major peaks are reported in cm^{-1} .

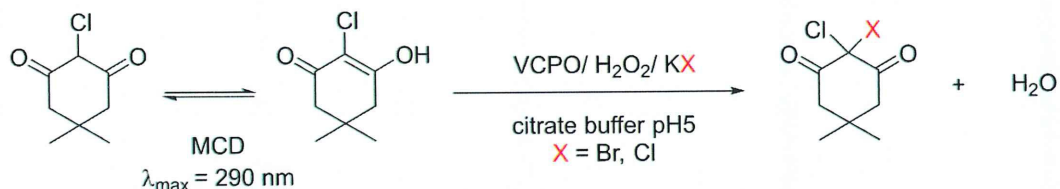
3.1.4 Plasmid

Oligonucleotides were purchased from Integrated DNA Technologies. The wild type and the variant genes of *Curvularia inaequalis* vanadium chloroperoxidase (CVCPO) were codon optimized and synthesized by Twist Bioscience (USA). Enzymes for molecular cloning were purchased from New England Biolabs. DNA purification kits were purchased from Vivantis. Sanger sequencing was conducted by MacroGen in Korea.

3.2 Protein Overexpression

Ten milliliters of an overnight culture of *E. coli* BL21(DE3) containing the overexpression plasmid of pET30-CVCPO-WT (wild type) or pET30-eCVCPO (variant) were inoculated into 1 liter of Luria-Bertani (LB) broth containing a final concentration of 50 µg/mL kanamycin. The culture was incubated at 37 °C with shaking at 200 rpm until the OD₆₀₀ reached approximately 0.6. The protein expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 200 µM. The protein was overexpressed as a fusion protein with an N-terminal His tag. The culture was then incubated at 20 °C with shaking at 200 rpm for an additional 16 hours. The cells were harvested by centrifugation at 5000 rpm at 8 °C for 25 minutes. The pellet was resuspended in lysis buffer (300 mM NaCl, 50 mM Na₂HPO₄, and 10 mM imidazole, pH 8.0). The cells were lysed in ice bath by sonication (1.5 s cycle, 50 % duty) for 30 seconds three times. The lysate was clarified by centrifugation at 12,000 rpm and 4 °C for 40 minutes. The supernatant was applied to a Ni-NTA column (QIAGEN) and the protein was purified according to the manufacturer's instructions. The purified protein was incubated with two equivalents of Na₃VO₄. Finally, the protein was desalted using a 10-DG column (BioRad) pre-equilibrated with the desalting buffer (100 mM potassium phosphate buffer, 20% glycerol, pH 7.5). The purified proteins were aliquoted and stored at -80 °C.

3.3 Monochlorodimedone assay (MCD assay)

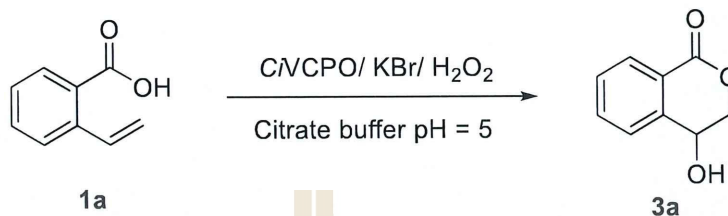


The consumption of MCD was monitored at 290 nm using a UV-Vis spectrophotometer. Each assay was performed in a total volume of 600 μL in a quartz cuvette with 1 cm path length. Before the reaction, the mixture consisted of 50 μM MCD, 1 mM H₂O₂, and 10 mM KBr or KCl in 100 mM citrate buffer (pH 5.0). The reaction was initiated by the addition of 1 μM of the enzyme and the reaction progress was recorded at 290 nm for every second.

3.4 *CVCPO* Michaelis–Menten kinetics

To determine the apparent kinetic parameters for the *CVCPO* wild-type and variant, the monochlorodimedone (MCD) assays were performed with varying concentrations of KBr. The reactions were conducted in 600 μL of 100 mM citrate buffer (pH 5.0) containing 50 μM MCD, 1 mM H₂O₂, and 1 μM of the *CVCPO* enzyme. The KBr concentrations used were 0.1, 0.3, 0.5, 1, 3, and 5 mM. The MCD consumption was monitored at 290 nm using a UV-Vis spectrophotometer, with measurements taken every 1 second over a period of 3 min. The initial reaction rates were calculated from the initial linear portion of the reaction curve. The MCD concentration in the assay was calculated using the extinction coefficient of MCD ($\epsilon = 20.2 \text{ mM}^{-1}\text{cm}^{-1}$). The apparent kinetic parameters, K_m and V_{max} were determined by fitting the initial reaction rates and substrate concentrations to the Michaelis–Menten equation using nonlinear regression in GraFit 5.0 (Erithacus Software, Horley, Surrey, UK). The apparent k_{cat} was calculated by dividing V_{max} by the enzyme concentration.

3.5 Optimizing the enzymatic halocyclization reaction of 2-vinylbenzoic acid



3.5.1 pH optimization experiments

The reactions were conducted using various buffers at different pH levels: H₂O, 100 mM acetate buffer (pH 3), 100 mM citrate buffer (pH 4), 100 mM citrate buffer (pH 5), 100 mM potassium phosphate buffer (pH 7), and 100 mM Tris buffer (pH 9). After the reactions, the mixtures were adjusted to pH 1 to 2, followed by extraction with CH₂Cl₂ three times and dried over Na₂SO₄. The solvent was removed by a rotary evaporator. The conversions and yields were quantified using trans-cinnamic acid as an internal standard, which was added prior to extraction.

3.5.2 KBr and H₂O₂ optimization experiments

Each reaction contained 40 mM of 2-vinylbenzoic acid (**1a**), CVCPO (400 nM), and different concentrations of KBr and H₂O₂ in 100 mM citrate buffer (pH 5). Each reaction was stirred at room temperature at 600 rpm for 24 hours. After the reaction, the mixture was adjusted to pH 1 to 2, followed by extraction with CH₂Cl₂ three times and dried over Na₂SO₄. The solvent was removed by a rotary evaporator. The conversion was quantified using an internal standard of trans-cinnamic acid, which was added before the extraction.

3.5.3 Reaction time experiments

Incubation times of 3, 6, 9, and 24 hours will be evaluated to determine the optimal reaction duration. Once the ideal conditions for the halocyclization of 2-vinylbenzoic acid are established, the best reaction conditions will be applied to test additional starting materials.

3.5.4 Cosolvent test experiments

Each reaction contained 40 mM of 5-methoxy-2-vinylbenzoic acid (**1b**), H₂O₂ (170 mM), KBr (160 mM), and CVCPO (400 nM) in 100 mM citrate buffer (pH 5) containing 30% organic solvent (DMSO, DMF, EtOH, or MeOH). Each reaction was stirred at room temperature at 600 rpm for 24 hours. After the reactions, the mixtures were

adjusted to pH 1 to 2, followed by extraction with CH_2Cl_2 three times and dried over Na_2SO_4 . The solvent was removed by a rotary evaporator. The conversion and yield were quantified using an internal standard of trans-cinnamic acid, which was added before the extraction. Every solvent test was conducted in triplicate.

3.6 Substrate scope and synthesis of 2-vinylbenzoic acid derivatives (1b-1h)

3.6.1 Substrate scope

The 8 substrates shown in the figure 3.1 were tested for cyclization reactions by the enzyme system.

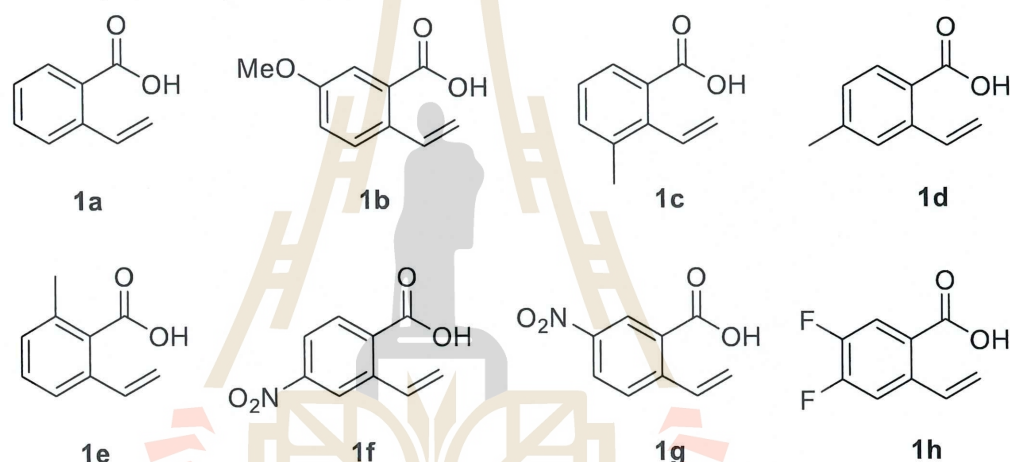
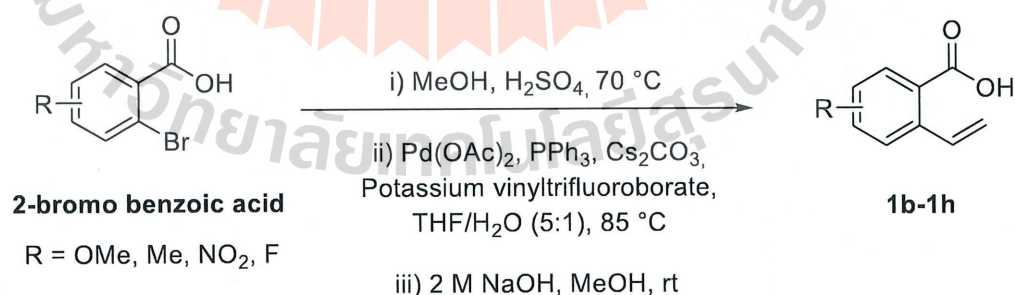


Figure 3.1 All substrates tested with cyclization reaction.

3.6.2 Synthesis of 2-vinylbenzoic acid derivatives (1b-1h)



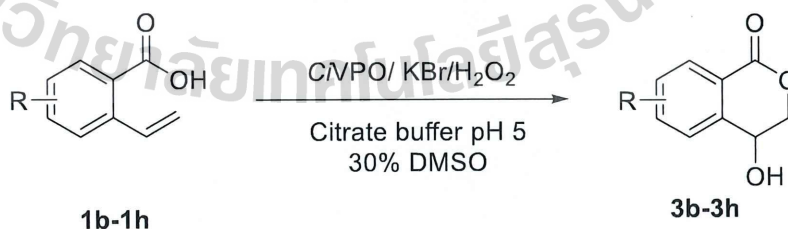
Step i: A 50 mL round-bottom flask equipped with a magnetic stir bar was used for the reaction. 2-Bromobenzoic acid (500 mg) was dissolved in MeOH (10 mL) and stirred for a few minutes. Then, concentrated H_2SO_4 (0.5 mL) was added to the mixture. The mixture was then refluxed for 12 hours. The reaction was monitored by thin-layer chromatography using a hexane and ethyl acetate system. After the reaction was

complete, the mixture was transferred to a separatory funnel containing H₂O and CH₂Cl₂. The aqueous layer was extracted with CH₂Cl₂ (3 x 20 mL). The combined organic phases were dried over Na₂SO₄ and concentrated under reduced pressure, yielding a product that was used directly in the next step.

Step ii: A 50 mL single-necked round-bottom flask under a nitrogen atmosphere, Pd(OAc)₂ (5 mol%), triphenylphosphine (14 mol%), Cs₂CO₃ (2.5 equiv.), and potassium vinyltrifluoroborate (1.3 equiv.) were added. A solution of methyl 2-bromobenzoate (1.0 equiv.) in anhydrous THF and H₂O (THF/H₂O = 5/1) was then added to the flask. The resulting suspension was stirred at 85 °C overnight. After completion of the reaction, the mixture was filtered through a pad of Celite, and the pad was washed with ether. The combined filtrate was washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using hexane/ethyl acetate as the eluent. The purified product was then used in the next step.

Step iii: The product from step ii was dissolved in 10 mL of methanol (MeOH), to which 5 mL of 2M NaOH solution was then added. The reaction mixture was stirred at room temperature for 16 hours. Subsequently, the resulting mixture was acidified with 1M HCl and extracted 20 mL of ethyl acetate (EtOAc) three times. The combined organic layers were dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure to afford the desired products, which were characterized by ¹H and ¹³C-NMR, and high-resolution mass spectroscopy.

3.7 The enzymatic halocyclization reaction of 2-vinylbenzoic acid derivative



All 2-vinylbenzoic acid derivatives **1b-1h** will undergo testing under the optimized conditions for the halocyclization reaction. The reaction will be extracted using CH₂Cl₃ in three sequential extractions of 5 mL. The combined organic layers were washed with brine and dried over Na₂SO₄. The evaporation of the organic solvent was carried out in the rotary evaporator at 50 °C. The reaction will be monitored, and both

the conversion and yield will be determined by ^1H -NMR spectroscopy with an external standard. The product will be characterized by ^1H -NMR, and ^{13}C -NMR, and confirmed by high-resolution mass spectrometry (HRMS).



CHAPTER IV

RESULTS AND DISCUSSION

4.1 Monochlorodimedone assay (MCD assay)

In the monochlorodimedone (MCD) assay used to investigate the kinetics of *CVCPO*, the variant demonstrated a significant enhancement in catalytic activity. Specifically, the variant exhibited a 6-fold increase in bromination and a 2-fold increase in chlorination at pH 5.0 compared to the wild-type enzyme (Hasan et al., 2006). Despite this notable improvement, the variant had not been utilized in any published biocatalytic reactions, prompting further investigation into its properties and potential advantages over the wild-type enzyme. To explore this, the *CVCPO* wild-type and the variant, both containing an N-terminal His-tag, were overexpressed in *E. coli* BL21(DE3) and purified using Ni-NTA chromatography. The protein yields for both the wild-type and the variant were comparable, indicating that the mutation did not adversely affect protein expression or stability.

The catalytic activities of the purified enzymes were then evaluated using the MCD assay, which monitors the disappearance of MCD at 290 nm via UV-Vis spectroscopy. The assay conditions included 1 mM H₂O₂, 50 μ M MCD, 1 μ M enzyme, and either 10 mM KCl (for chlorination) or KBr (for bromination) in 100 mM citrate buffer at pH 5.0. The results revealed that the variant oxidized both bromide and chloride at significantly faster rates than the wild-type enzyme (Figure 4.1). This aligns with the initial observations of enhanced bromination and chlorination activities, confirming the superior catalytic efficiency of the variant under the tested conditions. The variant's enhanced activity suggests that the mutation may have modified the active site or substrate-binding properties, improving halide oxidation. However, its lack of use in biocatalytic reactions raises concerns about its stability, substrate specificity, or performance in complex conditions. Further studies are needed to understand the mechanisms and assess its practical application.

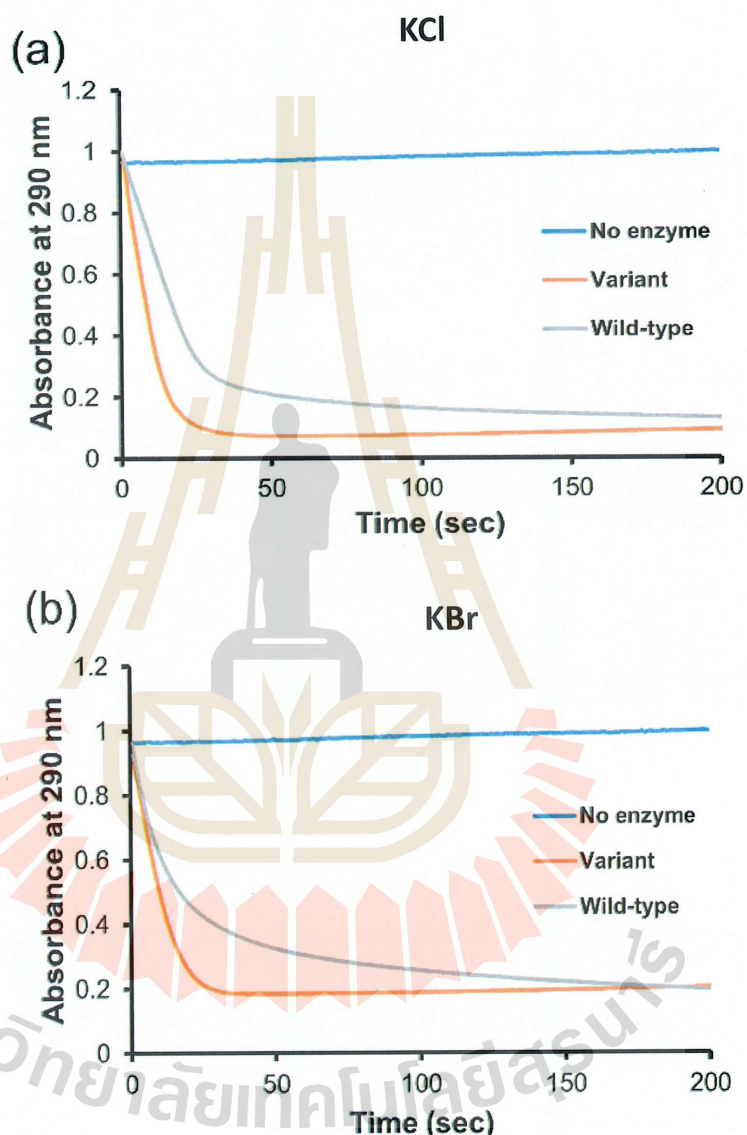
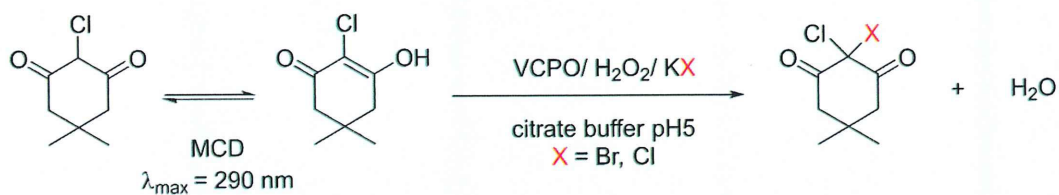


Figure 4.1 Activity comparisons of the VCPO wild-type and the variant using the MCD assay. The reaction mixture consisted of 1 mM H_2O_2 , 50 μM MCD, 1 μM enzyme, and either 10 mM KCl (a) or KBr (b) in 100 mM citrate buffer at pH 5.0.

4.2 *CVCPO* Michaelis–Menten kinetics

The Michaelis-Menten kinetic parameters of the *CVCPO* wild-type and variant were determined by varying bromide concentrations while maintaining a fixed concentration of H_2O_2 , *CVCPO*, and MCD (Figure 4.2). The variant exhibited a k_{cat} value of 50.90 s^{-1} , which is approximately 5-fold higher than that of the wild-type 10.86 s^{-1} . This significant increase in catalytic turnover suggests that the variant is more efficient in generating hypohalite, a key intermediate in the reaction.

Given that the activation of the alkene is considered the rate-limiting step, I hypothesize that the variant's enhanced ability to produce hypohalite could accelerate the overall halocyclization process. This improved catalytic efficiency makes the *CVCPO* variant a promising candidate for further investigation. Consequently, the variant will be utilized in subsequent studies to explore its potential in biocatalytic applications.

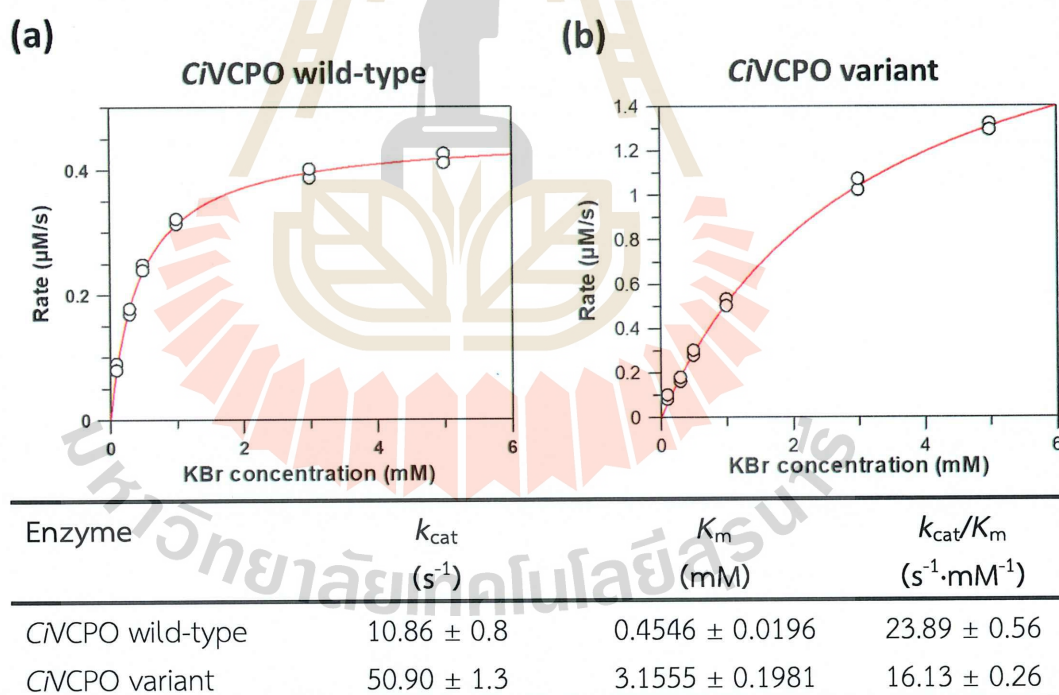
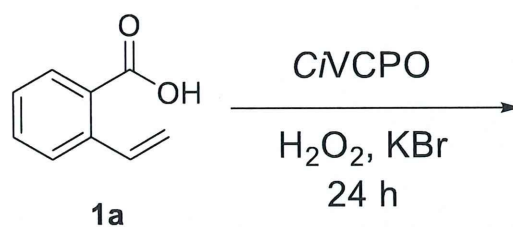


Figure 4.2 Apparent of kinetic parameters for *CVCPO* wild-type (a) and variant (b) with the fixed 1 mM H_2O_2 , 50 μM MCD, 1 μM enzyme, and varied concentrations of KBr.

4.3 Optimization of the enzymatic cyclization process for 2-vinylbenzoic acid

The study commenced with the cyclization of 40 mM 2-vinylbenzoic acid (**1a**) under a variety of reaction conditions, as detailed in Table 1. Initial experiments were designed based on previously reported optimized conditions, which utilized 160 mM KBr and 170 mM H₂O₂ (Dong et al., 2017). A range of pH conditions were systematically evaluated (entries 1–6), with pH 5 (entry 4) emerging as the most effective, achieving complete conversion of **1a** within 24 hours. This optimal pH aligns with findings from other studies involving CVCPO-catalyzed reactions, (Höfler et al., 2020; Naapuri et al., 2017; Younes et al., 2020) reinforcing its appropriateness for this specific transformation. Conversions were meticulously quantified using ¹H-NMR spectroscopy, with trans-cinnamic acid employed as an internal standard to ensure accuracy.

To enhance reagent efficiency and reduce potential waste, lower concentrations of KBr and H₂O₂ were explored (entries 7–12). However, these adjustments did not yield complete conversion, indicating that the original reagent concentrations were critical for achieving optimal results. Control experiments were conducted to underscore the necessity of each reaction component. These experiments demonstrated that the omission of the enzyme, KBr, or H₂O₂ resulted in no conversion (entries 13–15), highlighting the indispensable role of all components in the reaction system. Time-course analysis was performed to monitor the reaction progression, revealing a steady increase in product formation over time, with full conversion achieved within 15 hours (Figure 4.3). To ensure reproducibility and consistent results across all subsequent experiments, a 24-hour reaction time was adopted, providing a buffer to account for any minor variations in reaction conditions.

Table 4.1 Optimization of the cyclization conditions of 2-vinylbenzoic acid.

Entry	pH ^[a]	KBr (mM)	H ₂ O ₂ (mM)	Conversion ^[b] (%)
1	Water	160	170	12
2	3	160	170	25
3	4	160	170	30
4	5	160	170	Full
5	7	160	170	28
6	9	160	170	7
7	5	40	170	32
8	5	80	170	50
9	5	120	170	80
10	5	160	40	53
11	5	160	80	69
12	5	160	120	77
13 ^c	5	160	170	0
14	5	0	170	0
15	5	160	0	0

^[a] Buffer used: acetate (pH 3), citrate (pH 4 and 5), and Tris-HCl (pH 7 and 9).

^[b] Determined by ¹H-NMR for the crude reaction mixtures.

^[c] The reaction did not contain CVCPO.

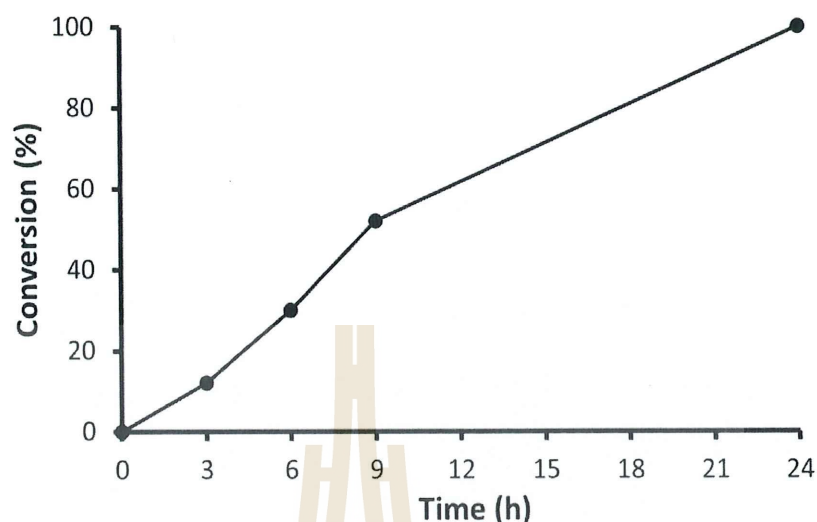


Figure 4.3 The time course of the chemoenzymatic cyclization of 2-vinylbenzoic acid (**1a**) was monitored under the following conditions: 40 mM **1a**, 160 mM KBr, 170 mM H₂O₂, and 400 nM CVCPO in 100 mM citrate buffer at pH 5. The conversion of **1a** was determined by measuring its concentration using ¹H-NMR spectroscopy. Each time point was conducted in triplicate to ensure reproducibility and accuracy of the results.

After the reaction was completed, the major product was identified using thin-layer chromatography (TLC) and ¹H-NMR analysis of the crude mixture. The product was then isolated and thoroughly characterized using ¹H-NMR, ¹³C-NMR, and high-resolution mass spectroscopy. Initially, based on a similar reaction mechanism involving NBS (Chen et al., 2012), the expected product was 4-bromoisochroman-1-one (**2**). However, the isolated product was unexpectedly identified as 4-hydroxyisochroman-1-one (**3a**). The presence of the hydroxyl group was further confirmed by Fourier-transform infrared (FT-IR) spectroscopy (Figure 4.4), which displayed distinct absorption bands at 1711 cm⁻¹ (carbonyl group) and 3391 cm⁻¹ (hydroxyl group). Given that CVCPO is known to release hypohalite from its active site to facilitate reactions, the enantioselectivity of the process was not investigated.

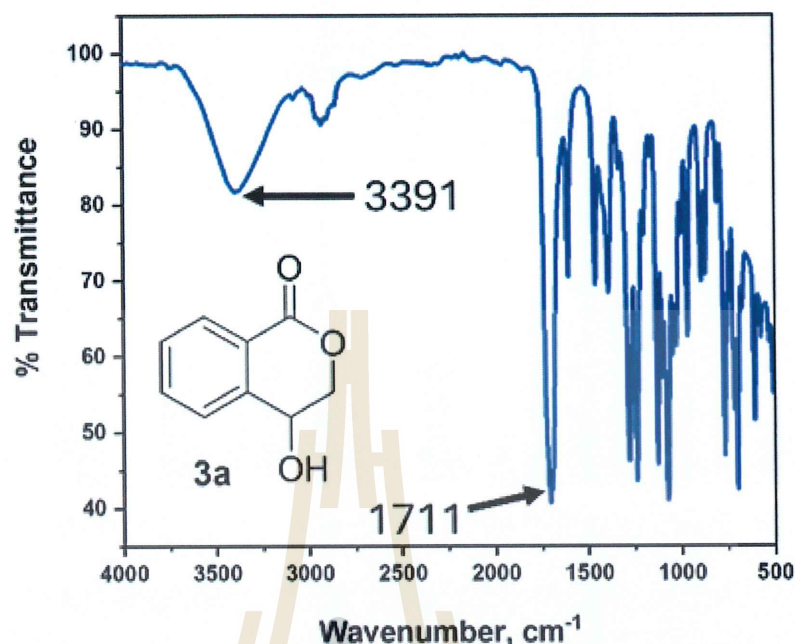


Figure 4.4 FT-IR analysis of the isolated product **3a**. The signals of 1711 and 3391 cm^{-1} correspond to the carbonyl and hydroxyl groups, respectively

In the cyclization reaction (Table 1, Entry 4), CVCPO catalyzed the generation of hypobromite through the oxidation of bromide using H_2O_2 . To compare this enzymatic approach with chemical methods for generating electrophilic bromine, two alternative reactions were tested for the cyclization of **1a** (Scheme 4.1). The first approach employed oxone/KBr, where oxone, a green oxidant containing potassium peroxymonosulfate, was used to oxidize bromide to hypobromite in a mixture of acetone and water (Scheme 1b) (Alvi et al., 2022; Rai et al., 2021). However, ^1H -NMR analysis of the crude reaction mixture revealed a relatively low yield of 29%

4.3.1 The cyclization of 2-vinylbenzoic acid under various

The second approach, conducted by a colleague from AK Lab, involved the use of NBS in the presence of triphenylphosphine sulfide (Ph_3PS) as a Lewis base catalyst (Figure 4.5c). This method drew inspiration from a previously reported reaction in which triphenylphosphine sulfide effectively catalyzed the cyclization of 5-phenyl-4-pentenoic acid, leading to the selective formation of the 6-endo cyclization product with a high endo/exo selectivity ratio of 91:1 (Denmark and Burk, 2010). However, in my case, the outcome differed significantly. TLC analysis of the reaction mixture revealed a complex array of products, and only the 5-exo cyclization product was successfully isolated, achieving a modest yield of 37%. This result contrasts with the

high selectivity observed in the reported reaction, suggesting that the substrate and reaction conditions play a critical role in determining the cyclization pathway and product distribution.

These experiments highlight the distinct advantages of the *CVCPO*-catalyzed cyclization. Unlike the chemical methods, the enzymatic approach provided a single 6-endo product with excellent yield and selectivity. This underscores the efficiency and potential of *CVCPO* as a green and effective catalyst for organic transformations, offering a superior alternative to traditional chemical reagents for product **3a**.

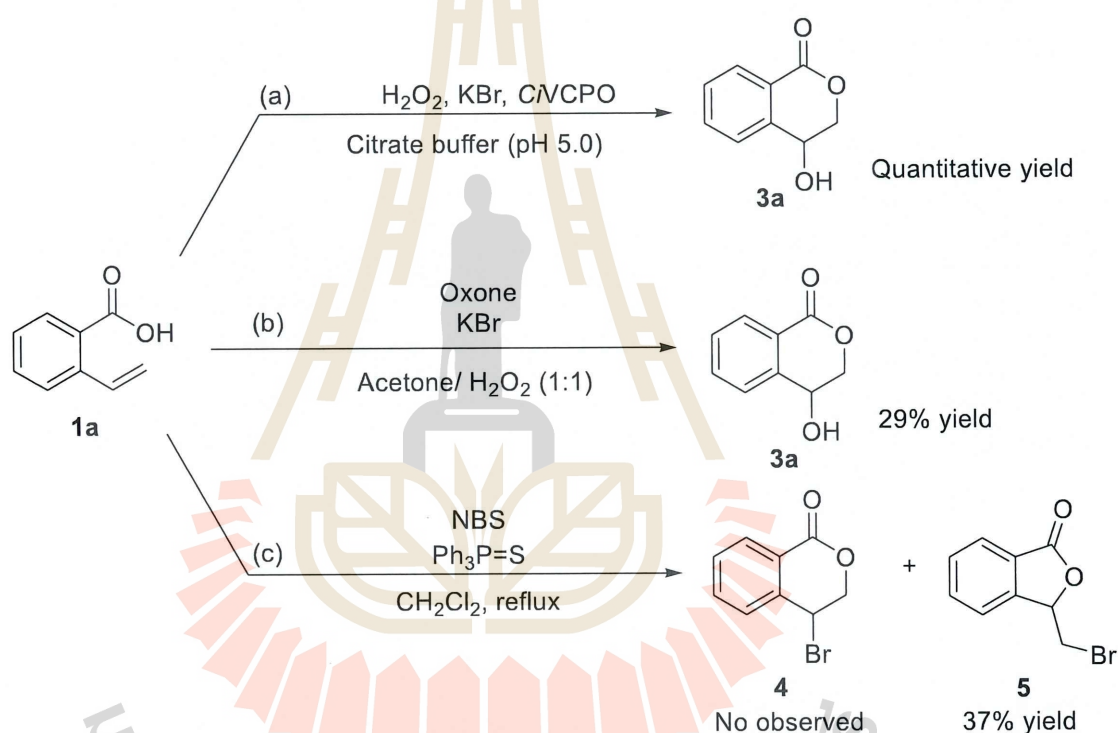


Figure 4.5 The cyclization of 2-vinylbenzoic acid (**1a**) under various conditions over a 24-hour period. (a) The reaction is catalyzed by *CVCPO*. (b) Oxone oxidizes KBr to produce hypobromite, which facilitates the cyclization. (c) NBS drives the cyclization in the presence of a Lewis base catalyst ($\text{Ph}_3\text{P}=\text{S}$).

4.4 Co-solvent test

To further explore the potential of the reaction for synthesizing various 4-hydroxyisochroman-1-one derivatives, First, synthesized 5-methoxy-2-vinylbenzoic acid (**1b**) as a substrate. However, during the initial setup of the biocatalytic reaction, we observed that substrate **1b** exhibited poor solubility in the citrate buffer, resulting in a significantly low reaction yield of only 14% (Figure 4.6). To address this issue, we investigated the use of organic cosolvents, which have been previously employed in other *CVCPO*-catalyzed reactions to enhance substrate solubility and reaction efficiency (Wells et al., 2023).

I tested several common organic solvents, including DMSO, DMF, EtOH, and MeOH, at a final concentration of 30% in the reaction mixture. The results demonstrated that the addition of 30% DMSO significantly improved the solubility of substrate **1b**, leading to a dramatic increase in the reaction yield. Under these optimized conditions, the corresponding product **3b** was obtained in an 82% yield, which was notably higher than the yields achieved with other cosolvents (Figure 4). This finding highlights the critical role of solvent selection in biocatalytic reactions, particularly for substrates with limited aqueous solubility. The use of DMSO as a cosolvent not only enhanced the reaction efficiency but also underscored the versatility of *CVCPO* in accommodating diverse reaction conditions for the synthesis of valuable organic compounds.

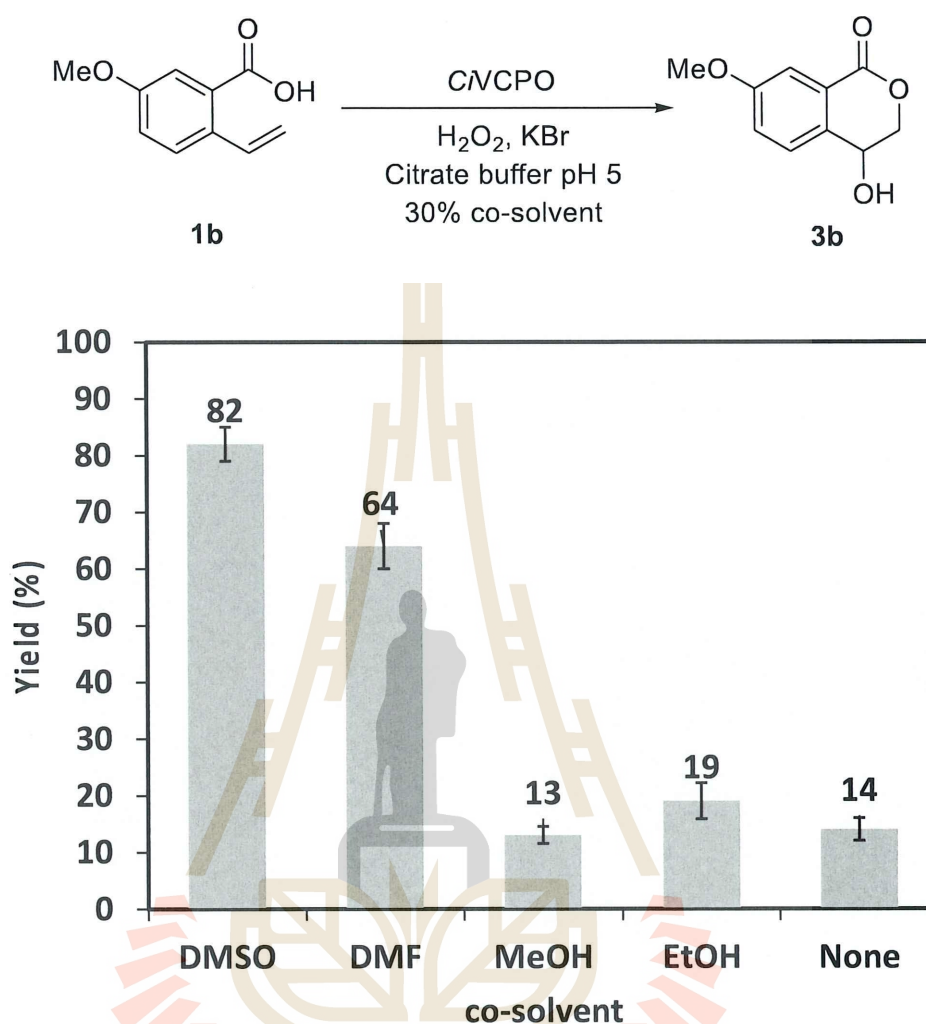


Figure 4.6 The cyclization of compound **1b** catalyzed by *CVCPO* in the presence of a 30% cosolvent. The reaction conditions comprised 40 mM of **1b**, 160 mM KBr, 170 mM H_2O_2 , and 400 nM *CVCPO* in a 100 mM citrate buffer with 30% cosolvent. The yields of the resulting product **3b** were quantified through 1H -NMR analysis. Each solvent test was performed in triplicate.

4.5 Analog test

The incorporation of 30% DMSO enhanced substrate solubility without losing the enzymatic activity of *CVCPO*. To assess the broader applicability of our catalytic system, I synthesized and tested a series of substrates with different substituents (Figure 4.7). The yields of the reactions were quantified using 1H -NMR analysis. The

findings revealed that substrates **1b–1e**, featuring electron-donating groups, were efficiently converted into their respective 4-hydroxyisochroman-1-one derivatives (**3b–3e**). These products were successfully isolated and characterized using ^1H -NMR, ^{13}C -NMR, and high-resolution mass spectrometry (HRMS). In contrast, substrates **1f**, **1g**, and **1h**, which contain electron-withdrawing groups, did not undergo cyclization. This failure is likely due to the diminished electron density of the vinyl group caused by the inductive effects of the electron-withdrawing groups, which hinder the electrophilic addition of hypobromite (Dong et al., 2017).

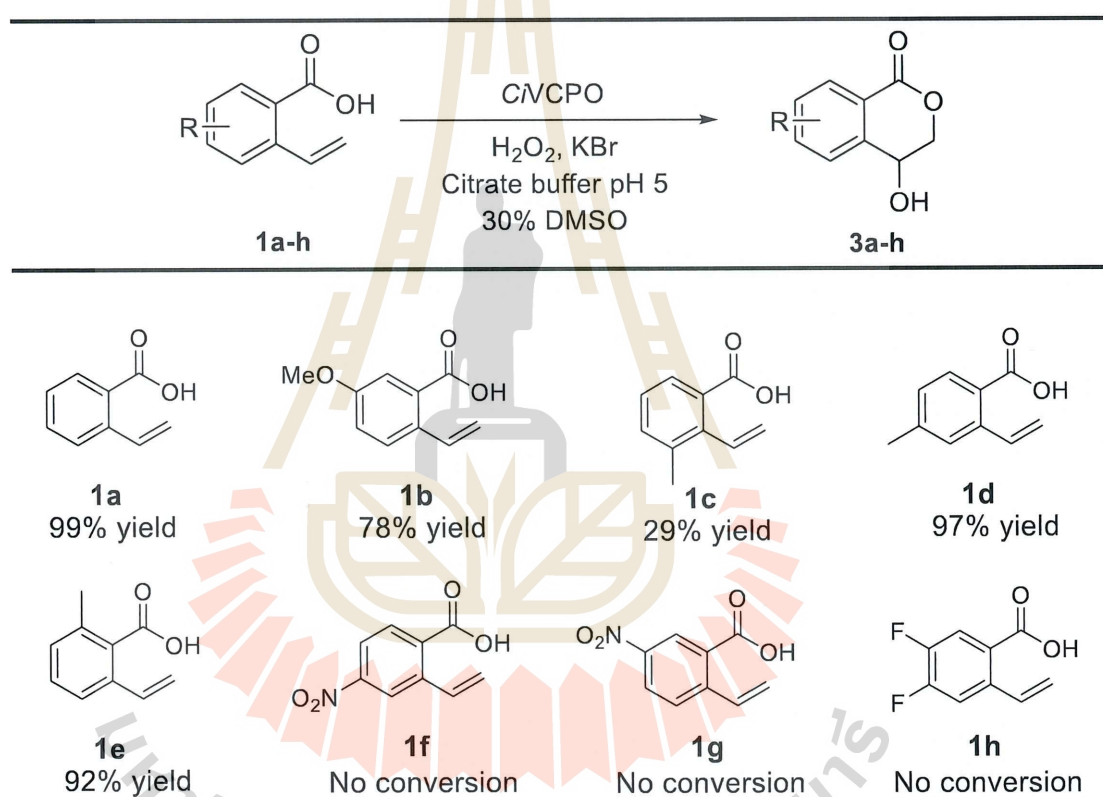


Figure 4.7 The substrate scope for the CVCPO-catalyzed cyclization of 2-vinylbenzoic acids (**1a–1h**). The reaction conditions included 40 mM substrate, 160 mM KBr, 170 mM H_2O_2 , and 400 nM CVCPO in a 100 mM citrate buffer with 30% DMSO, conducted over 24 hours. The yields of the reactions were determined using ^1H -NMR analysis.

Initially, I expected the formation of the brominated product **4** rather than the hydroxyl product (**3a**) (Scheme 4.1). However, the substrate scope experiments (Scheme 4.2) yielded only the hydroxyl products (**3a–3e**). Furthermore, NMR analysis

of the crude reaction mixture from the time course study showed no detectable byproducts. This result is consistent with prior observations in the cyclization of 4-pentenoic acid, where both hydroxylactone and bromolactone were produced (Höfler et al., 2020).

4.6 Proposed mechanism for formation of the hydroxyl product

To explain these observations, I propose a mechanism for the formation of the hydroxyl product **3a** from substrate **1a** (Figure 4.8). The reaction begins with CVCPO catalyzing the generation of hypobromite, which facilitates the electrophilic addition to substrate **1a**, forming the bromonium intermediate **6**. This intermediate undergoes hydrolysis to produce *vic*-halohydrin **7**, which exists in equilibrium with the corresponding epoxide **8**. The carboxylate group then intramolecularly attacks the epoxide, leading to the formation of the hydroxyl product **3a** (Höfler et al., 2020). This proposed mechanism elucidates the observed product formation and underscores the pivotal roles of hypobromite and intermediate equilibria in the reaction pathway.

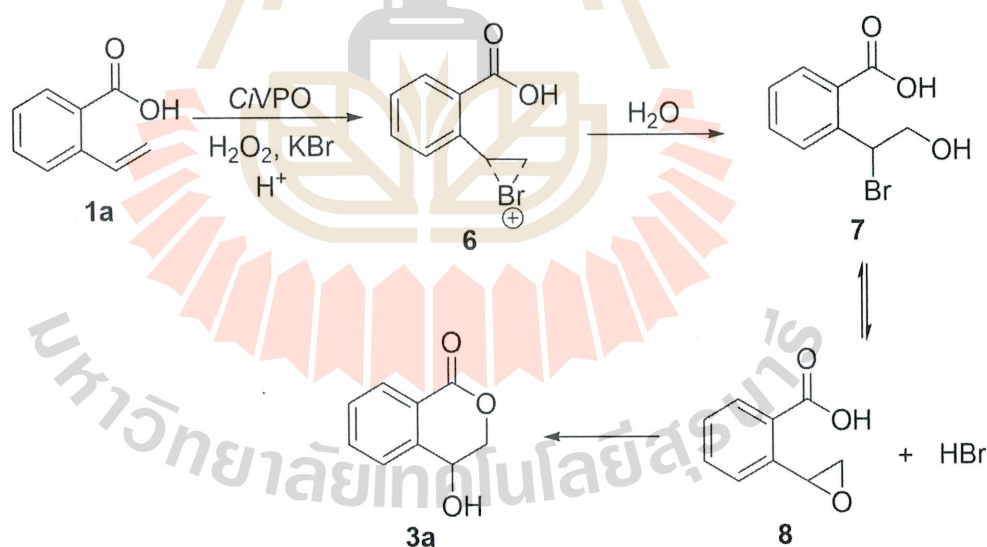


Figure 4.8 Proposed mechanism for formation of the hydroxyl product **3a** from substrate **1a**.

4.7 References

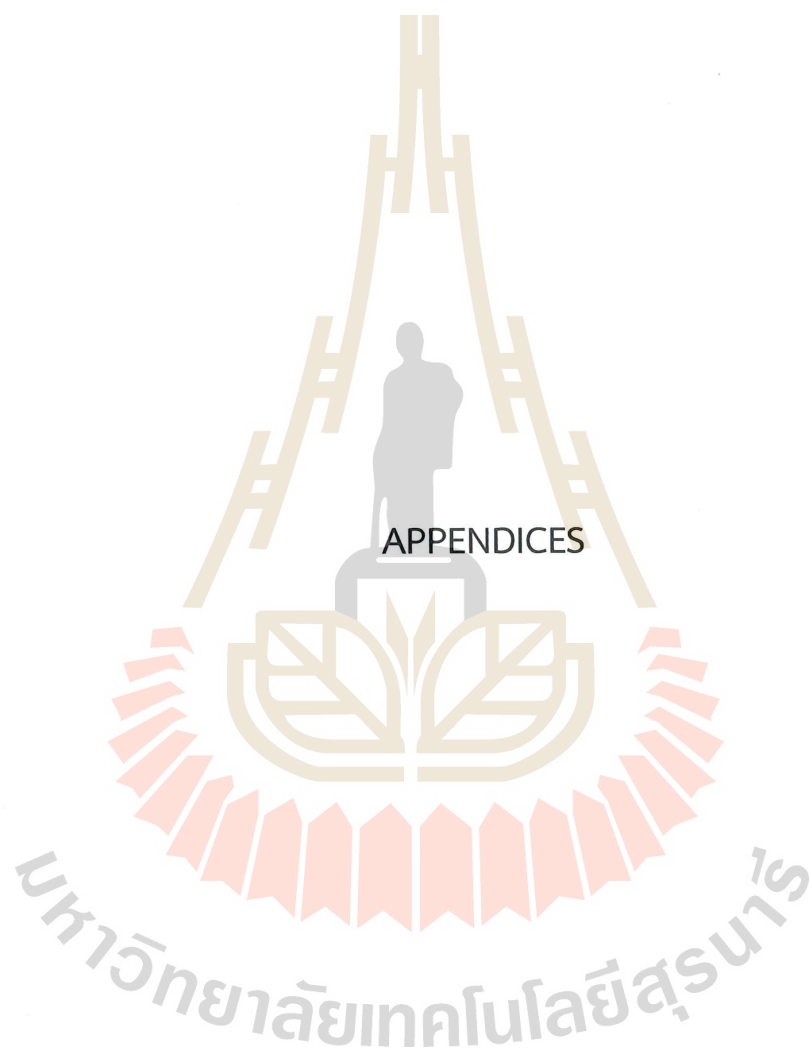
- Alvi, S., Jayant, V., and Ali, R. (2022). Applications of oxone® in organic synthesis: an emerging green reagent of modern era. *ChemistrySelect*. 7(23), e202200704.
- Chen, J., Zhou, L., Tan, C. K., and Yeung, Y.-Y. (2012). An enantioselective approach toward 3, 4-dihydroisocoumarin through the bromocyclization of styrene-type carboxylic acids. *The Journal of Organic Chemistry*. 77(2), 999-1009.
- Denmark, S. E., and Burk, M. T. (2010). Lewis base catalysis of bromo- and iodolactonization, and cycloetherification. *Proceedings of the National Academy of Sciences*. 107(48), 20655-20660.
- Dong, J. J., Fernández-Fueyo, E., Li, J., Guo, Z., Renirie, R., Wever, R., and Hollmann, F. (2017). Halofunctionalization of alkenes by vanadium chloroperoxidase from *Curvularia inaequalis*. *Chemical Communications*. 53(46), 6207-6210.
- Hasan, Z., Renirie, R., Kerkman, R., Ruijsenaars, H. J., Hartog, A. F., and Wever, R. (2006). Laboratory-evolved vanadium chloroperoxidase exhibits 100-fold higher halogenating activity at alkaline pH: catalytic effects from first and second coordination sphere mutations. *Journal of Biological Chemistry*. 281(14), 9738-9744.
- Höfler, G. T., But, A., Younes, S. H., Wever, R., Paul, C. E., Arends, I. W., and Hollmann, F. (2020). Chemoenzymatic halocyclization of 4-pentenoic acid at preparative scale. *ACS sustainable chemistry & engineering*. 8(7), 2602-2607.
- Naapuri, J., Rolfes, J. D., Keil, J., Sapu, C. M., and Deska, J. (2017). Enzymatic halocyclization of allenic alcohols and carboxylates: a biocatalytic entry to functionalized O-heterocycles. *Green Chemistry*. 19(2), 447-452.
- Rai, V., Sorabad, G. S., and Maddani, M. R. (2021). Transition metal free, green and facile halogenation of ketene dithioacetals using a KX-oxidant system. *New Journal of Chemistry*. 45(2), 1109-1113.
- Wells, C. E., Ramos, L. P., Harstad, L. J., Hessefort, L. Z., Lee, H. J., Sharma, M., and Biegasiewicz, K. F. (2023). Decarboxylative Bromooxidation of Indoles by a Vanadium Haloperoxidase. *ACS Catalysis*. 13(7), 4622-4628.
- Younes, S. H., Tieves, F., Lan, D., Wang, Y., Süß, P., Brundiek, H., Wever, R., and Hollmann, F. (2020). Chemoenzymatic halocyclization of γ , δ -unsaturated carboxylic acids and alcohols. *ChemSusChem*. 13(1), 97-101.

CHAPTER V

CONCLUSIONS

In summary, a chemoenzymatic cyclization process for 2-vinylbenzoic acids was successfully developed, enabling the exclusive production of 4-hydroxyisochroman-1-ones via the 6-endo pathway. While the substrate scope is currently limited to compounds containing electron-donating groups, the method demonstrates remarkable selectivity and efficiency. To highlight the advantages of this approach, two conventional chemical methods were employed for comparison. The results revealed that the CVCPO-catalyzed cyclization outperformed traditional methods, offering superior yields and selectivity. Notably, this biocatalytic process eliminates the generation of toxic byproducts, aligning with the principles of green chemistry. This breakthrough not only represents a significant advancement in synthetic methodology but also underscores the potential of enzymatic catalysis as a sustainable and environmentally friendly alternative.





APPENDIX A

PROTEIN PURIFICATION AND PRODUCT CHARACTERIZATION

A.1 Plasmid and sequence information of vanadium-chloroperoxidase

A1.1 DNA sequence of *CVCPO* wild type:

ATGGGATCAGTAACACCTATACCACTACCCAAGATCGATGAGCCGGAAGAGTACAATA
CCAACCTATATCCTGTTCTGGAATCATGTTGGCTTAGAGTTGAATCGTGTTACCCACACCGTGGG
TGGTCCGTTGACGGGTCCGCCATTAAGTGCGCGCGCACTCGGTATGCTGCATCTGGCGATCCAC
GACGCGTATTTCTCTATCTGTCCGCCGACCGATTTACCACGTTTCTGAGCCCGGATACCGAAA
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TGCGAACATTTCCGATAACGCCTACGCCAGCTGGGTTTGGTTCTGGATCGTTCTGTTCTCGAA
GCACCGGGCGGTGTTGATCGTGAAAGCGCAAGCTTTATGTTTGGTGAAGACGTTGCGGACGTGT
TCTTCGCGTTGCTGAACGATCCGCGTGGTGCGAGCCAAGAGGGTTATCACCCGACGCCGGGTC
GCTACAAGTTTGACGATGAGCCAACCCATCCGGTCGTGTTGATCCCGGTGGACCCAAACAACCC
GAATGGCCCCAAAAATGCCGTTTCGCCAATATCACGCCCCATTCTACGGCAAACACCAAACGT
TTTGCGACCCAGAGCGAGCACTTTCTTGAGATCCGCCGGGTCTTCGGTCCAACGCAGACGAGA
CGGCTGAATACGACGACGCGGTTTCGCGTGGCCATCGCCATGGGTGGCGCGCAAGCGCTGAACT
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TGATCGGTACCCACCGCGTTTTTATAACCAGATTGTGCGCCGTATCGCGGTCACCTATAAGAA
GGAGGAGGATTTGGCAAATTCTGAAGTGAACAACGCGGACTTCGCGCGTTTGTTTGGCGTGGTT
GACGTGGCCTGCACAGATGCCGGTATCTTCAGCTGGAAAGAAAAGTGGGAATTTGAGTTCTGGC
GTCCGCTGTCCGGTGTTCGCGACGACGGGCGTCCAGATCACGGCGATCCGTTCTGGCTAACCTT
GGGCGCACCGGCGACGAACACCAACGATATCCCATTCAAACTCCGTTCCCGGCGTACCCGAGC
GGACATGCGACCTTCGGTGGTGCGGTGTTCCAAATGGTTAGACGTTACTATAATGGCCGTGTGG
GCACCTGGAAGGATGACGAGCCGGACAACATTGCGATTGACATGATGATCTCCGAAGAGTTGAA
TGGTGTTAATCGTGATCTGCGTCAACCTTATGATCCGACGGCCCCCATTGAGGACCAGCCGGGC
ATCGTTTCGACCCGTATTGTTTCGTCACTTCGACAGCGCATGGGAACTTATGTTTCGAGAACGCGA
TTAGCCGCATCTTCTTAGGCGTGCATTGGCGTTTTGACGCGGCGGCTGCTCGTGATATTCTGAT
TCCGACTACTACGAAGGATGTTTATGCTGTGGATAATAACGGCGCTACCGTTTTTCAGAACGTC

GAGGACATCCGTTACACCACTCGCGGTACCCGTGAAGACCCGGAGGGCCTCTTTCCGATCGGC
GGCGTACCGCTGGGCATCGAGATTGCAGATGAAATTTTAAACAACGGTCTGAAACCGACTCCGC
GGAGATTAGCCGATGCCGCAAGAAACCCCTGTCCAGAAACCGGTCGGTCAGCAGCCGGTGAA
AGGTATGTGGGAGGAAGAACAAGCCCCGGTGGTTAAGGAAGCGCCGtaa

A1.2 Amino acid sequence of *CVCPO* wild type (The His-tag sequence is underlined.):

MSSHHHHHHSSGENLYFOGGMGSVTP IPLPKIDEPEEYNTNYILFWNHVGLNELNRVTHT
VGGPLTGPPLSARALGMLHLAIHDAYFSICPPTDFTTFLSPDTENAAYRLPSPNGANDARQAVAGAA
LKMLSSLYMKPVEQPNPNPGANISDNAYAQLGLVDRSVLEAPGGVDRESASFMGEDVADVFFAL
LNDPRGASQEGYHPTPGRYKFDDEPTHPVVLIPVDPNPNPGPKMPFRQYHAPFYGKTTKRFATQSE
HFLADPPGLRSNADETAEYDDAVRVAIAMGGAQALNSTKRSPWQTAQGLYWAYDGSNLIGTPPRF
YNQIVRRIAVTYKKEEDLANSEVNNADFARLFALVDVACTDAGIFSWKEKWEFEFWRPLSGVRDDGR
PDHGDPFWLTLGAPATNTNDIPFKPPFPAYPSGHATFGGAVFQMVRYYNGRVGTWKDDEPDNIAI
DMMISEELNGVNRDLRQPYDPTAPIEDQPGIVRTRIVRHFD SAWELMFENAI SRIFLGVHWRFDAAA
ARDILIPTTTTKD VYAVDNNGATVFQNVEDIRYTTTRGTREDPEGLFPIGGVPLGIEIADEIFNNGLKPTTP
EIQPM PQETPVQKPVGQQPVKGMWEEEQAPVVKEAP

A1.3 DNA sequence of *CVCPO* variant:

ATGGGATCAGTAACACCTATACCACTACCCAAGATCGATGAGCCGGAAGAGTACAATA
CCA ACTATATCCTGTTCTGGAATCATGTTGGCTTAGAGTTGAATCGTGTTACCCACACCGTGGG
TGGTCCGTTGACGGGTCCGCCATTAAGTGCGCGCGCACTCGGTATGCTGCATCTGGCGATCCAC
GACGCGTATTTCTCTATCTGTCCGCCGACCGATTTACCACGTTTCTGAGCCCGGATACCGAAA
ATGCAGCTTACCGCCTGCCGTGCCGAACGGCGCTAACGACGCCAGACAAGCCGTGGCGGGCG
CGGCTCTGAAAATGCTGTCTAGCCTGTACATGAAACCGGTAGAGCAGCCCCAACCCGAATCCGGG
TGCGAACATTTCGGATAACGCCTACGCCGAGCTGGGTTTGGTTCTGGATCGTTCTGTTCTCGAA
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GACGTGGCCTGCGCAGATGCCGGTATCTTCAGCTGGAAAGAAAAGTGGGAATTTGAGTTCTGGC
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 CGGACATGCGACCTTCGGTGGTGCGGTGTTCCAAATGTTAGACGTTACTATAATGGCCGTGTG
 GGCACCTGGAAGGATGACGAGCCGGACAACATTGCGATTGACATGATGATCTCCGAAGAGTTGA
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 CATCGTTCGCACCCGTATTGTTTCGTCACTTCGACAGCGCATGGGAACCTTATGTTTCGAGAACGCG
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 CGAGGACATCCGTTACACCACTCGCGGTACCCGTGAAGACCCGGAGGGCCTCTTTCGGATCGG
 CGGCGTACCGCTGGGCATCGAGATTGCAGATGAAATTTTTAACAACGGTCTGAAACCGACTCCG
 CCGGAGATTAGCCGATGCCGCAAGAAACCCCTGTCCAGAAACCGGTCCGTCAGCAGCCGGTG
 AAAGGTATGTGGGAGGAAGAACAAGCCCCGGTGGTTAAGGAAGCGCCGtaa

A1.4 Amino acid sequence of *CIVCPO* variant (e*CIVCPO*) (The His-tag sequence is underlined and the mutations are labeled with a red color.):

MSSHHHHHSSGENLYFOGGMGSVTPIPLPKIDEPEEYNTNYILFWNHVGLNELNRVHT
 VGGPLTGPPLSARALGMLHLAIHDAYFSICPPTDFTTFLSPDTENAAAYRLPSPNGANDARQAVAGAA
 LKMLSSLYMKPVEQPNPNPGANISDNAYAQLGLVLDRSVLEAPGGVDRESASFMGEDVADVFFAL
 LNDPRGASQEGYHPTPGRYKFDEPTHPVVLIPVDNPNNGPKMPFRQYHAPFYGKTTKRFATQSE
 HFVADPPGLRSNADETAEYDDAVRVAIAMGGAQALNSTKRSPWQTAQGLYWAYDGSNLIGTPPRF
 YNQIVRRIAVTYKKEEDLANSEVNNADFARLFALVDVACADAGIFSWKEKWEFEFWRPLSGVRDDGR
 PDHGDPFWLTLGAPATNTNDIPFKDPFPAYPSGHATFGGAVFQMVRYYNNGRVGTWKDDEPDNIAI
 DMMISEELNGVNRLRQPYDPTAPIEDQPGIVRTRIVRHFDSEAWELMFENISRIFLGVHWRFDAAA
 ARDILIPTTTKDVYAVDNNGATVFQNVEDIRYTRGTREDPEGLFPIGGVPLGIEIADEIFNNGLKPTTP
 EIQPMPEQETPVQKPVGQQPVKGMWEEEQAPVWKEAP

A.2 *CIVCPO* Purification

Ten milliliters of an overnight *E. coli* BL21(DE3) culture harboring pET30-*CIVCPO*-WT or pET30-e*CIVCPO* were inoculated into 1 L of LB broth with 50 µg/mL kanamycin. The culture was grown at 37 °C, 200 rpm until OD₆₀₀ ≈ 0.6, then induced with 200 µM IPTG and incubated at 20 °C, 200 rpm for 16 hours. Cells were harvested by centrifugation (5000 rpm, 8 °C, 25 minutes), resuspended in lysis buffer (300 mM NaCl, 50 mM Na₂HPO₄, 10 mM imidazole, pH 8.0), and lysed by sonication on ice (1.5 s cycle,

50% duty, 3 × 30 seconds). The lysate was clarified by centrifugation (12,000 rpm, 4 °C, 40 minutes), and the supernatant was purified using a Ni-NTA column. The protein was incubated with two equivalents of Na_3VO_4 , desalted using a 10-DG column (pre-equilibrated with 100 mM potassium phosphate buffer, 20% glycerol, pH 7.5), aliquoted, and stored at -80 °C.

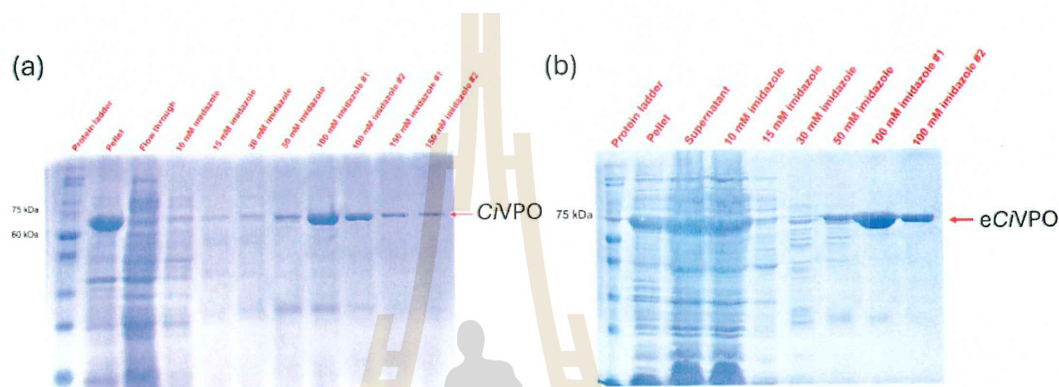
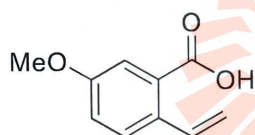
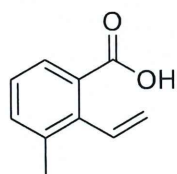


Figure A.1 10% SDS-PAGE analysis of CVCPO wild-type (a) and variant (b) purification.

A.3 Characterization of substrates

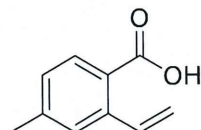


5-Methoxy-2-vinylbenzoic acid (1b): white solid (0.152 g, 30% yield), m.p. 103-104 °C; R_f 0.27 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.56 – 7.50 (m, 3H), 7.10 (dd, J = 8.7, 2.8 Hz, 1H), 5.59 (d, J = 17.3 Hz, 1H), 5.30 (d, J = 11.1 Hz, 1H), 3.86 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 173.0, 158.8, 135.4, 133.3, 128.8, 128.0, 119.9, 115.2, 115.2, 55.5.

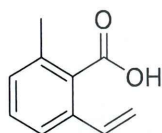


3-Methyl-2-vinylbenzoic acid (1c): white solid (0.175 g, 35% yield), m.p. 80-81 °C; R_f 0.28 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.76 (d, J = 7.7 Hz, 1H), 7.37 (d, J = 7.7 Hz, 1H), 7.22 (dd, J = 16.0, 8.0 Hz, 1H), 7.03 (dd, J = 17.9, 11.5 Hz, 1H), 5.49 (d,

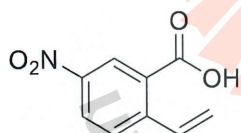
$J = 17.4$ Hz, 1H), 5.22 (d, $J = 11.0$ Hz, 1H), 2.35 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 173.5, 139.8, 136.9, 135.2, 134.6, 134.4, 128.1, 126.7, 119.1, 20.9.



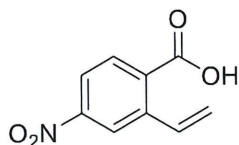
4-Methyl-2-vinylbenzoic acid (1d): white solid (0.165 g, 33% yield), m.p. 102-103 °C; R_f 0.43 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.97 (d, $J = 8.0$ Hz, 1H), 7.6 (dd, $J = 17.4, 10.9$ Hz, 1H), 7.41 (s, 1H), 7.17 (dd, $J = 8.2, 2.0$ Hz, 1H), 5.65 (d, $J = 16.2$ Hz, 1H), 5.36 (d, $J = 10.5$ Hz, 1H), 2.42 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 172.6, 143.9, 140.8, 136.3, 131.5, 128.4, 128.3, 124.3, 116.4, 21.6.



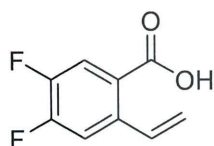
6-Methyl-2-vinylbenzoic acid (1e): colorless oil (0.063 g, 12% yield); R_f 0.19 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.43 (d, $J = 7.8$ Hz, 1H), 7.30 (t, $J = 7.7$ Hz, 1H), 7.15 (d, $J = 7.6$ Hz, 1H), 6.94 (dd, $J = 17.4, 10.9$ Hz, 1H), 5.74 (d, $J = 17.3$ Hz, 1H), 5.35 (d, $J = 10.9$ Hz, 1H), 2.43 (s, 3H).



5-Nitro-2-vinylbenzoic acid (1f): yellow solid (0.190 g, 38% yield), m.p. 143-145 °C⁴; R_f 0.13 (50% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 8.45 (s, 1H), 8.17 (d, $J = 1.4$ Hz, 2H), 7.55 (dd, $J = 17.4, 11.0$ Hz, 1H), 5.87 (d, $J = 17.4$ Hz, 1H), 5.58 (d, $J = 11.0$ Hz, 1H). ^{13}C NMR (126 MHz, CDCl_3) δ 169.1, 150.4, 142.1, 133.9, 132.5, 132.1, 122.3, 121.8, 119.9.

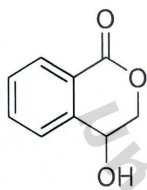


4-Nitro-2-vinylbenzoic acid (1g): yellow solid (0.200 g, 40% yield), m.p. 100-103 °C; R_f 0.10 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 8.46 (s, 1H), 8.18 (m, 2H), 7.56 (dd, $J = 17.4, 11.0$ Hz, 1H), 5.87 (d, $J = 17.4$ Hz, 1H), 5.59 (d, $J = 11.0$ Hz, 1H). ^{13}C NMR (126 MHz, CDCl_3) δ 170.1, 150.5, 142.1, 133.9, 132.5, 132.1, 122.3, 121.9, 119.9.

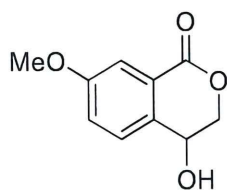


4,5-Difluoro-2-vinylbenzoic acid (1h): yellow solid (0.162 g, 32% yield), m.p. 123-124 °C; R_f 0.18 (50% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.89 (dd, $J_{\text{HF}} = 10.9, 8.1$ Hz, 1H), 7.53 (dd, $J_{\text{HF}} = 16.7, 10.2$ Hz, 1H), 7.39 (dd, $J = 11.3, 7.6$ Hz, 1H), 5.63 (d, $J = 17.4$ Hz, 1H), 5.43 (d, $J = 11.0$ Hz, 1H). ^{13}C NMR (125 MHz, CDCl_3) δ 169.8, 153.3 (dd, $J_{\text{CF}} = 250.8, 12.9$ Hz), 149.1 (dd, $J_{\text{CF}} = 250.8, 12.9$ Hz), 139.0 (q, $J_{\text{CF}} = 4.1$ Hz), 134.3, 123.2 (q, $J_{\text{CF}} = 4.1$ Hz), 120.7-120.6 (dd, $J_{\text{CF}} = 18.9, 2.1$ Hz), 118.1, 116.5 (d, $J_{\text{CF}} = 18.0$ Hz).

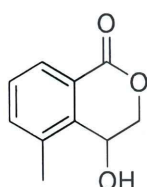
A.4 Product characterization



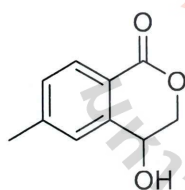
4-Hydroxyisochroman-1-one (3a): colorless liquid (5.8 mg, 99% yield), R_f 0.20 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 8.06 (dd, $J = 7.8, 1.4$ Hz, 1H), 7.65 (td, $J = 7.5, 1.4$ Hz, 1H), 7.52 (d, $J = 8.1$ Hz, 1H), 7.49 (td, $J = 7.6, 1.3$ Hz, 1H), 4.90 (dd, $J = 5.1, 3.4$ Hz, 1H), 4.56-4.48 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 164.6, 140.7, 134.4, 130.4, 129.4, 126.5, 123.8, 71.9, 64.2.



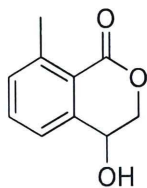
4-Hydroxy-7-methoxyisochroman-1-one (3b): colorless liquid (5.7 mg, 73.3% yield), R_f 0.46 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.51 (s, 1H), 7.40 (d, J = 8.4 Hz, 1H), 7.15 (d, J = 11.1, 1H), 4.81 (t, J = 3.6 Hz, 1H), 4.56-4.47 (m, 2H), 3.83 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 164.6, 160.3, 132.8, 128.4, 125.0, 122.0, 113.1, 72.5, 63.8, 55.7. HRMS (ESI): m/z $[\text{M} + \text{Na}^+]$ calcd for $\text{C}_{10}\text{H}_{10}\text{NaO}_4^+$ 217.0471, found 217.0471.



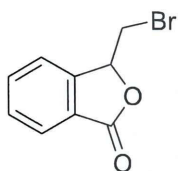
4-Hydroxy-5-methylisochroman-1-one (3c): colorless liquid (2.7 mg, 28.5% yield), R_f 0.22 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.99 (d, J = 7.7 Hz, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.41 (dd, J = 15.5, 8.0 Hz, 1H), 4.90 (s, 1H), 4.72 (d, J = 12.3 Hz, 1H), 4.54 (d, J = 10.2 Hz, 1H), 2.50 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 165.2, 137.5, 136.4, 136.1, 129.3, 128.0, 124.4, 72.7, 61.5, 17.9. HRMS (ESI): m/z $[\text{M} + \text{Na}^+]$ calcd for $\text{C}_{10}\text{H}_{10}\text{NaO}_3^+$ 201.0522, found 201.0522.



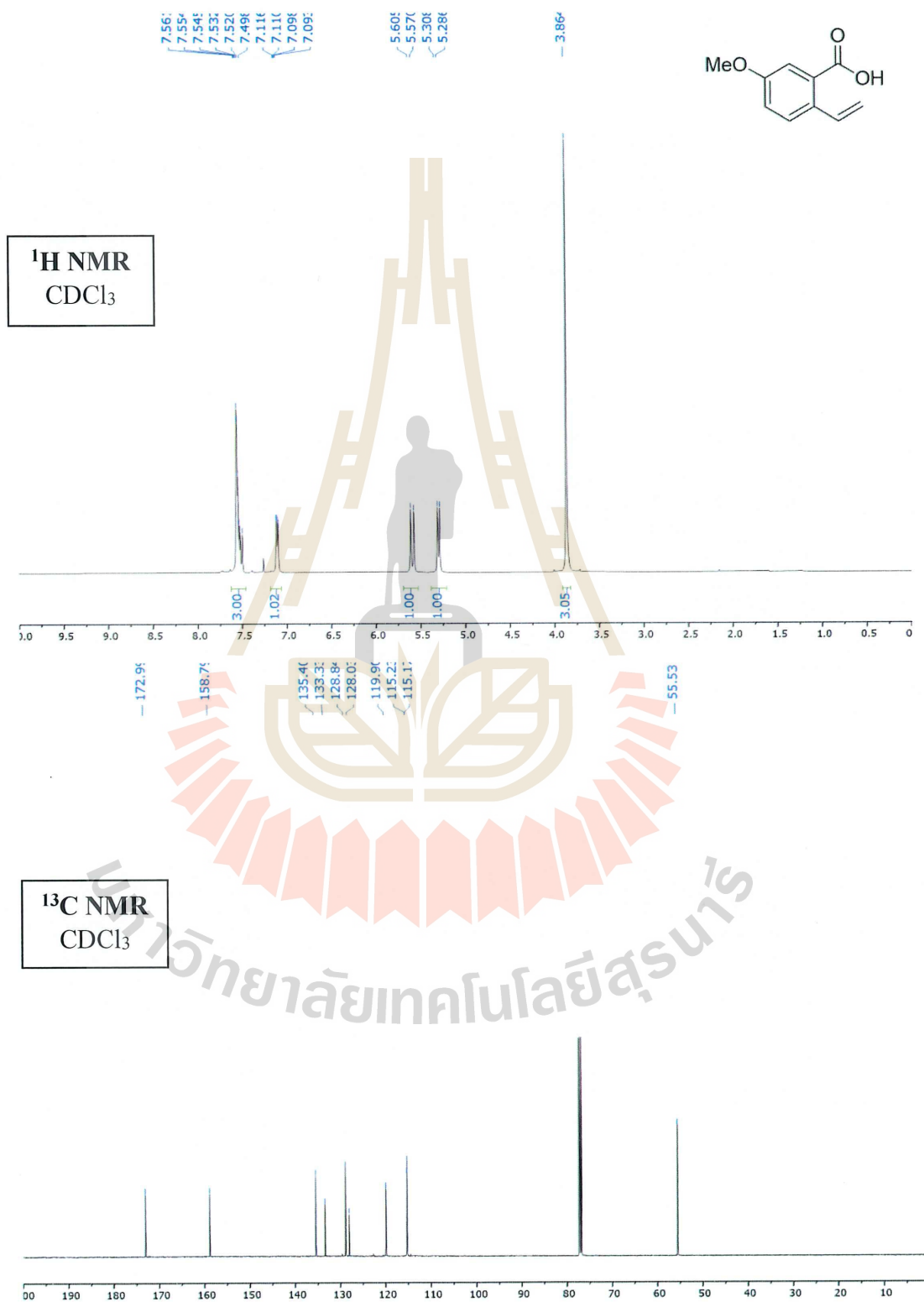
4-Hydroxy-6-methylisochroman-1-one (3d): colorless liquid (9.4 mg, 96.7% yield), R_f 0.21 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.90 (d, J = 7.9 Hz, 1H), 7.33 (s, 1H), 7.26 (d, J = 7.9, 1H), 4.85 (dd, J = 5.3, 3.5 Hz, 1H), 4.55 – 4.37 (m, 2H), 2.42 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 165.1, 145.7, 140.8, 130.3, 130.1, 127.0, 121.0, 72.0, 64.1, 21.9. HRMS (ESI): m/z $[\text{M} + \text{Na}^+]$ calcd for $\text{C}_{10}\text{H}_{10}\text{NaO}_3^+$ 201.0522, found 201.0522.



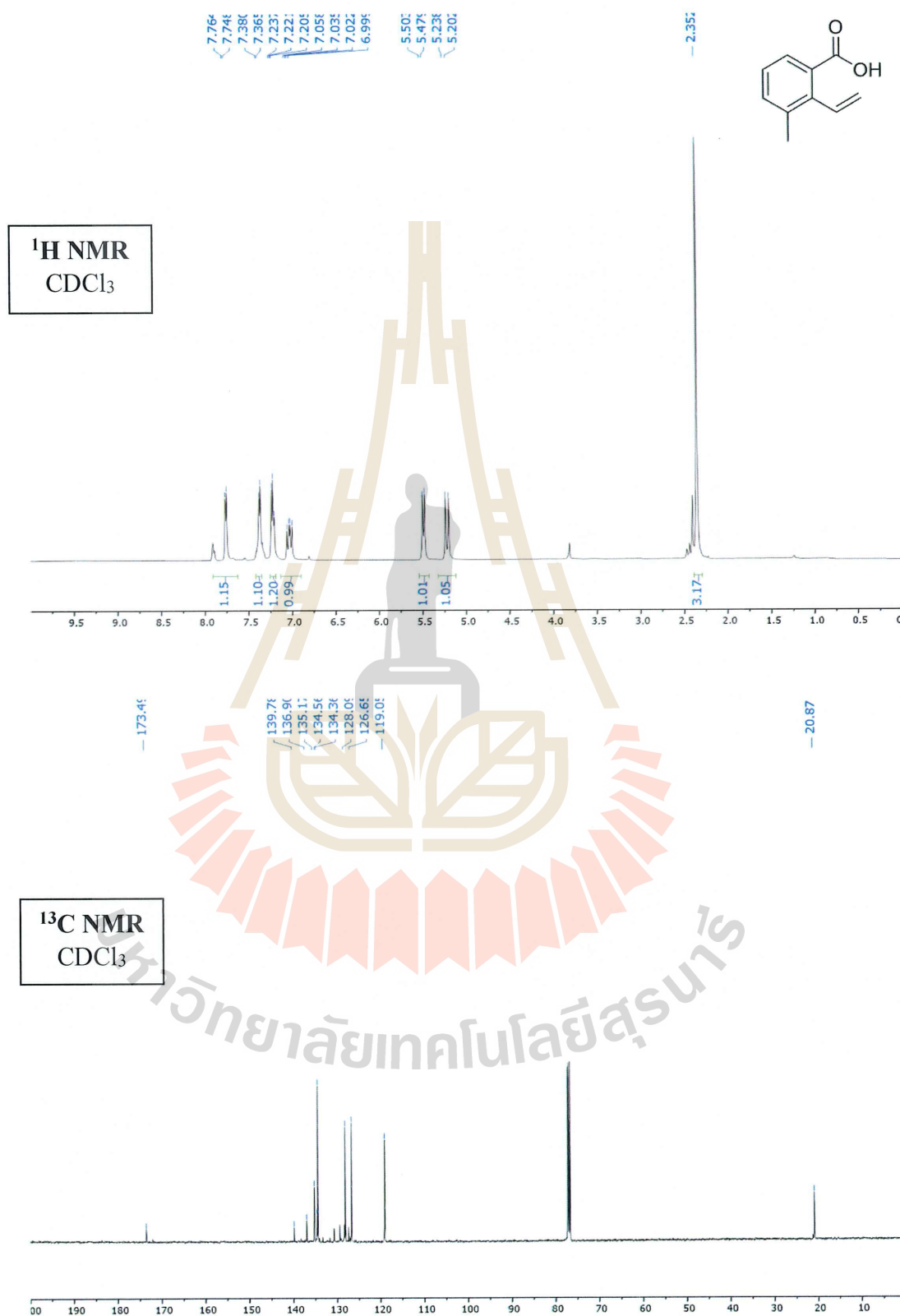
4-Hydroxy-8-methylisochroman-1-one (3e): colorless liquid (8.9 mg, 91.6% yield), R_f 0.46 (30% EtOAc/hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.49 (t, $J = 7.6$ Hz, 1H), 7.36 (d, $J = 7.6$ Hz, 1H), 7.31 (d, $J = 7.7$ Hz, 1H), 4.86 (dd, $J = 5.2, 3.2$ Hz, 1H), 4.49 – 4.40 (m, 2H), 2.65 (s, 3H). ^{13}C NMR (125MHz, CDCl_3) δ 164.3, 143.6, 142.2, 133.8, 133.2, 124.6, 122.5, 71.5, 65.4, 22.5. HRMS (ESI): m/z $[\text{M} + \text{Na}^+]$ calcd for $\text{C}_{10}\text{H}_{10}\text{NaO}_3^+$ 201.0522, found 201.0522.



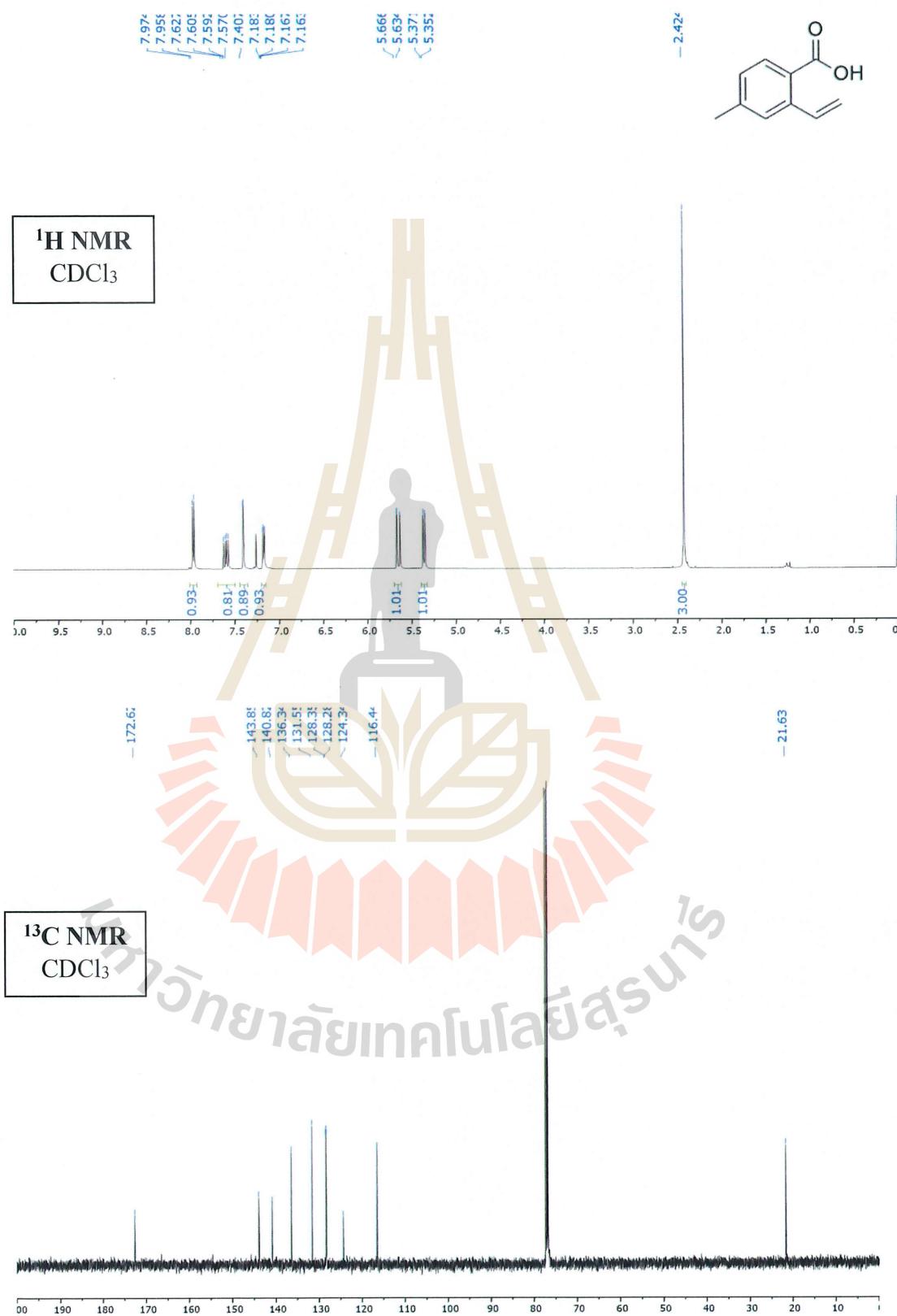
3-(Bromomethyl)-1(3H)-isobenzofuranone: white solid (0.0210 g, 37% yield); mp 69-71 °C; R_f 0.34 (20% EtOAc/ hexanes); ^1H NMR (500 MHz, CDCl_3) δ 7.95 (d, $J = 7.6$ Hz, 1H), 7.74 (t, $J = 7.5$ Hz, 1H), 7.69 – 7.57 (m, 2H), 5.72 (t, $J = 5.1$ Hz, 1H), 3.79 (d, $J = 5.1$ Hz, 2H). ^{13}C NMR (125MHz, CDCl_3) δ 169.4, 147.2, 134.3, 130.0, 126.5, 125.9, 122.4, 78.6, 32.1.

A.5 ^1H and ^{13}C -NMR data*5-Methoxy-2-vinylbenzoic acid (1b):*

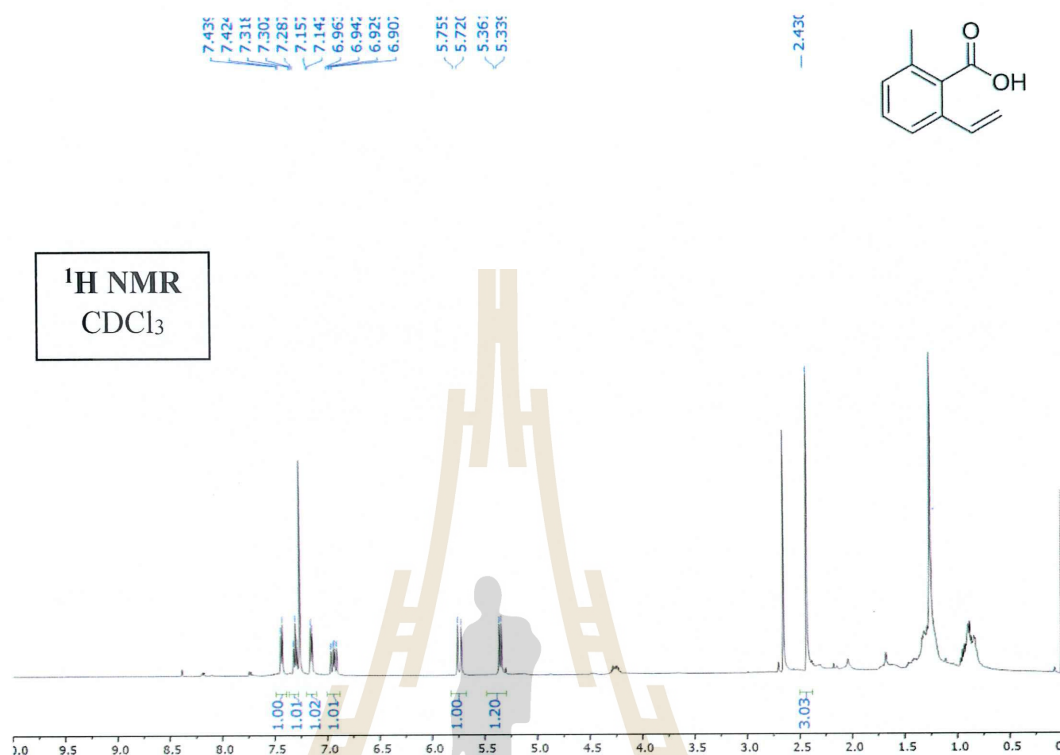
3-Methyl-2-vinylbenzoic acid (1c):



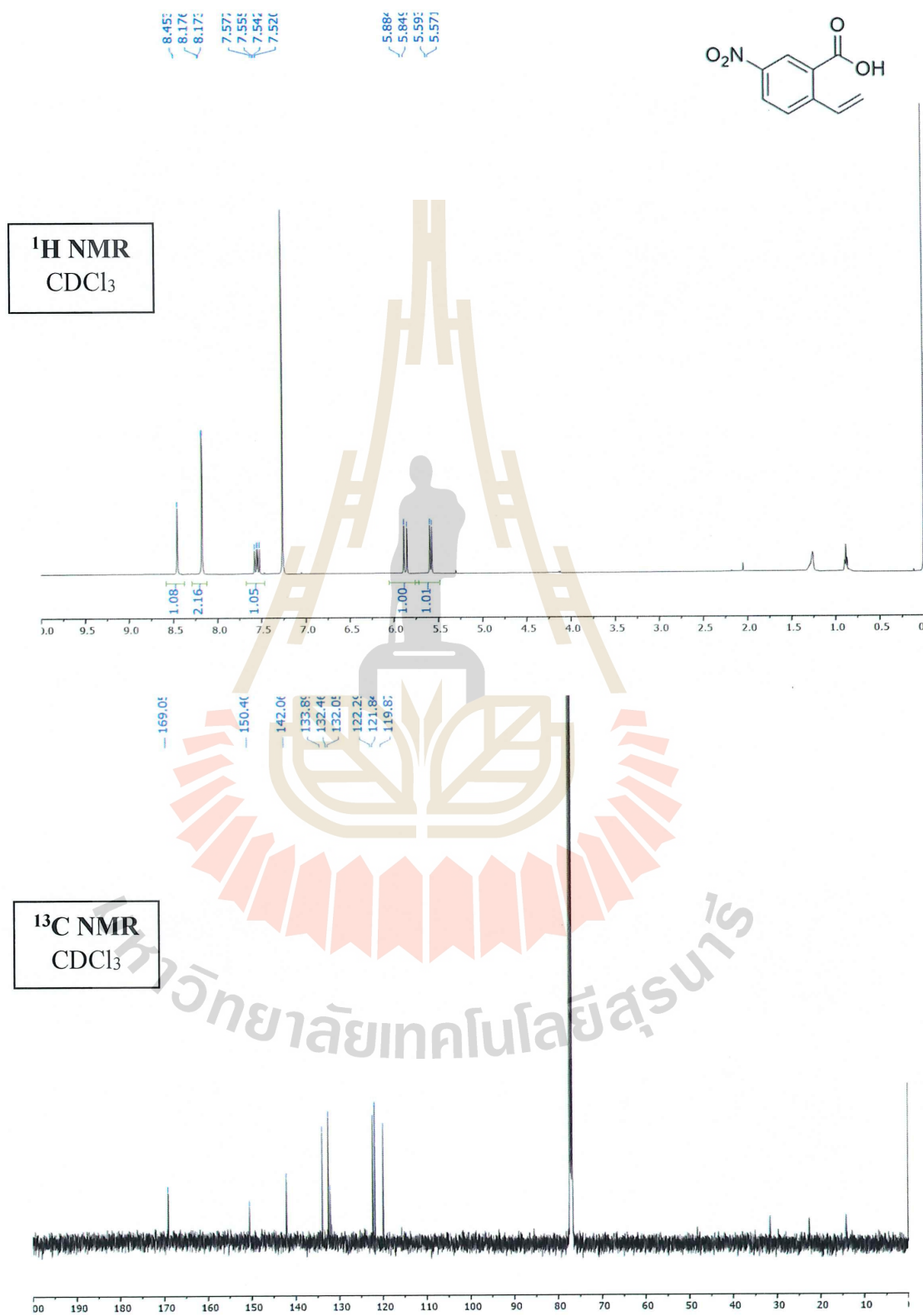
4-Methyl-2-vinylbenzoic acid (**1d**):



6-Methyl-2-vinylbenzoic acid (**1e**):

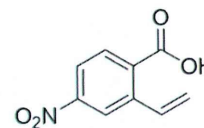


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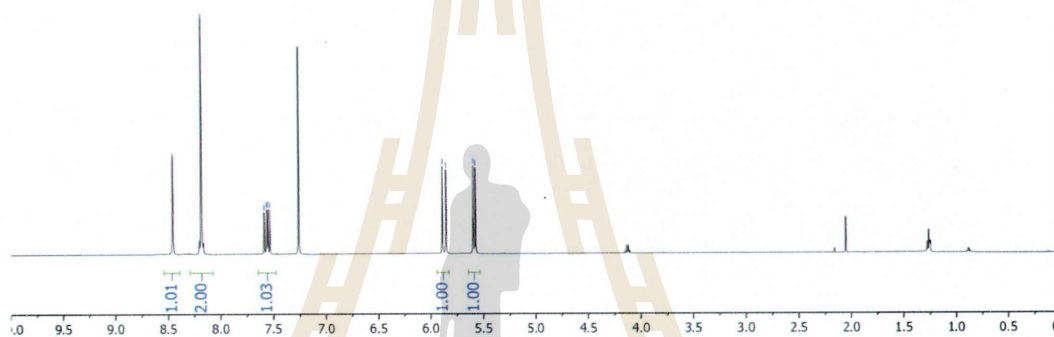
5-Nitro-2-vinylbenzoic acid (1f)

4-Nitro-2-vinylbenzoic acid (**1g**)

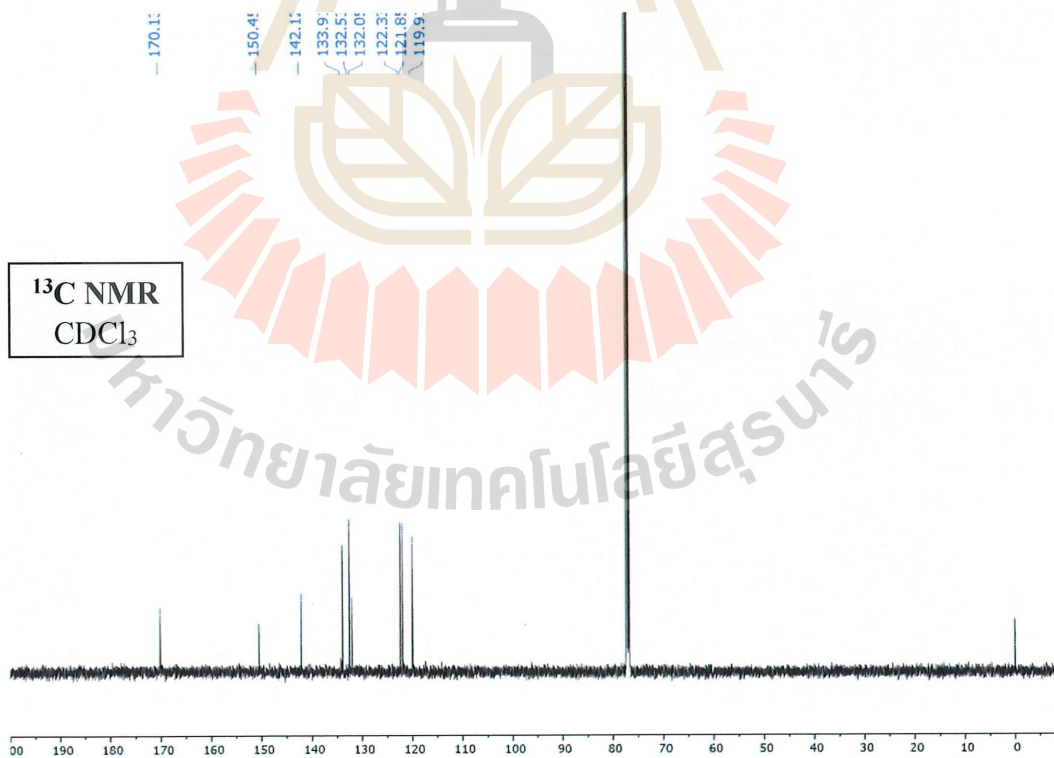
8.456
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5.575



¹H NMR
CDCl₃

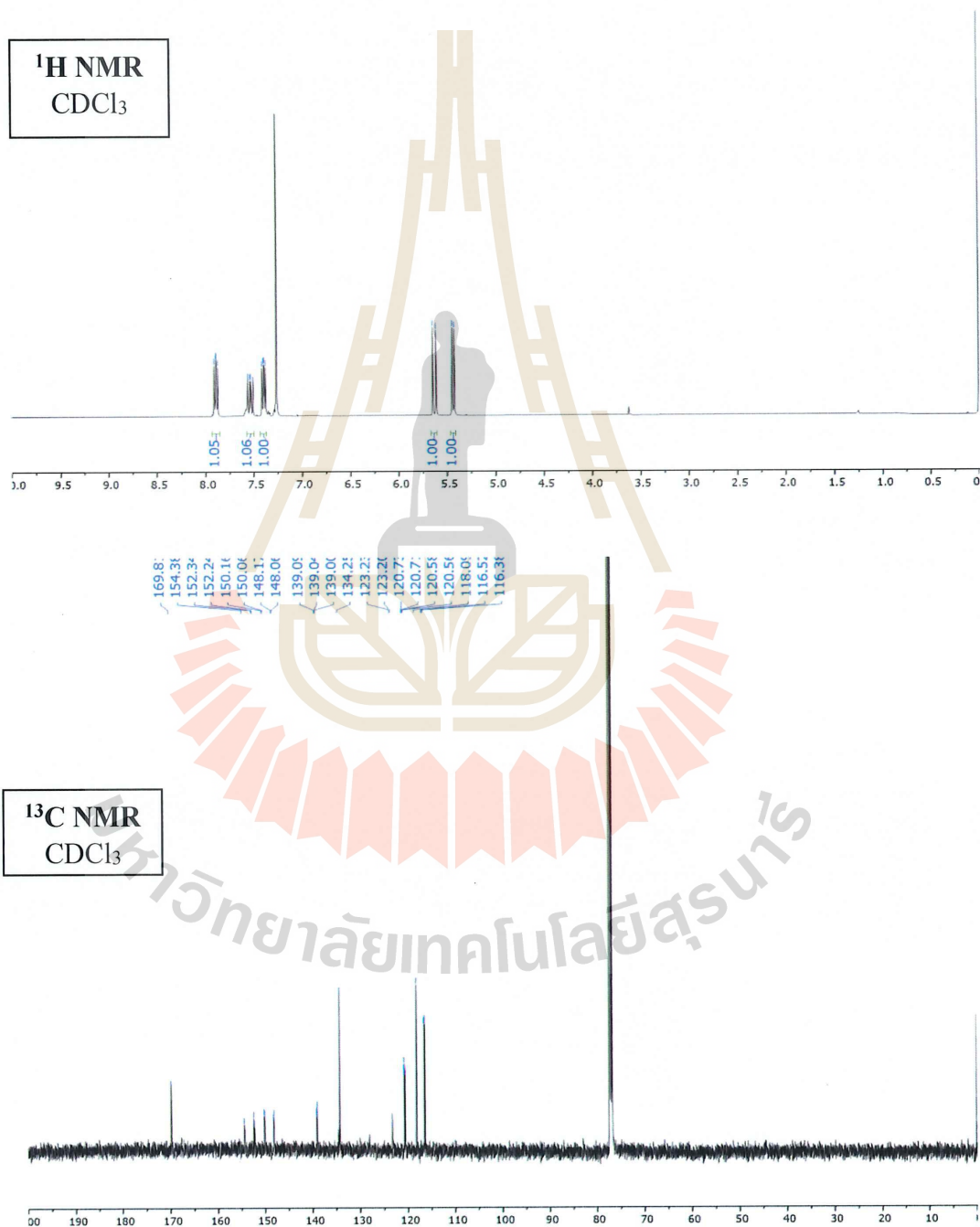
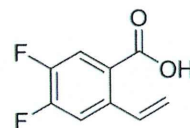


¹³C NMR
CDCl₃

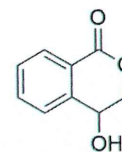


4,5-Difluoro-2-vinylbenzoic acid (1h)

7.911, 7.895, 7.890, 7.874, 7.561, 7.539, 7.528, 7.506, 7.411, 7.398, 7.375, 5.644, 5.611, 5.449, 5.421

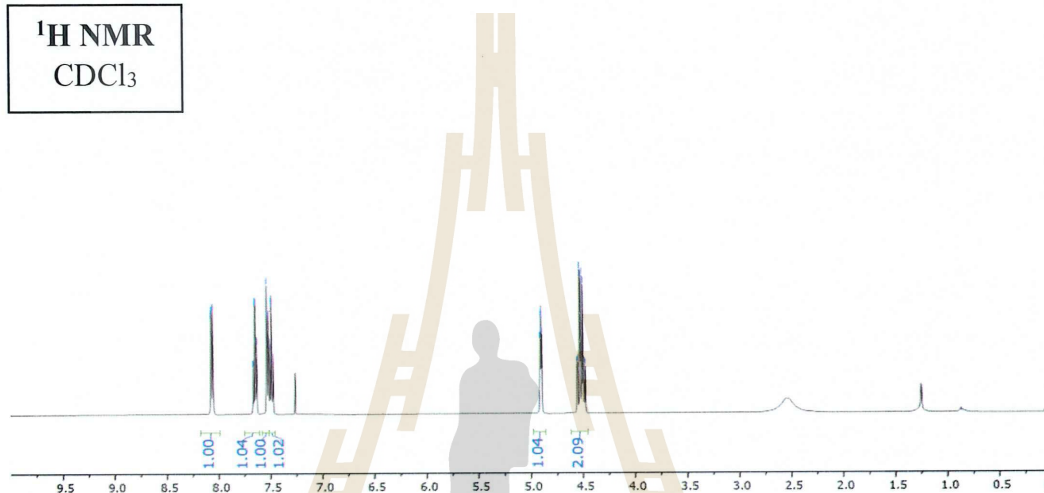


4-Hydroxyisochroman-1-one (3a)

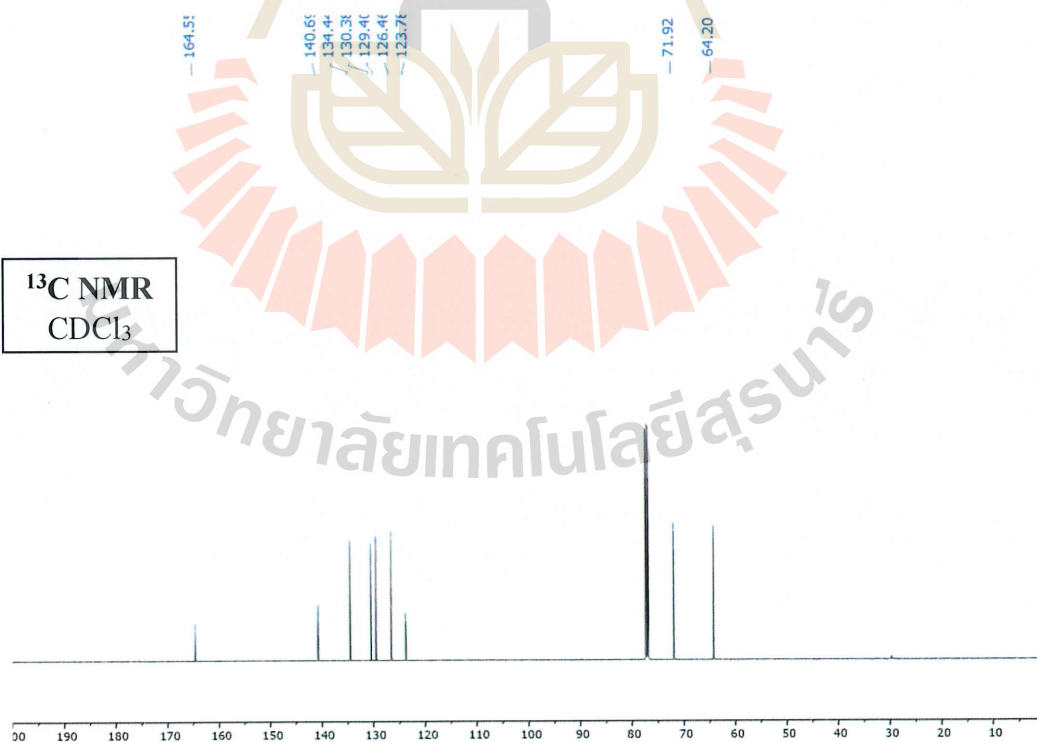


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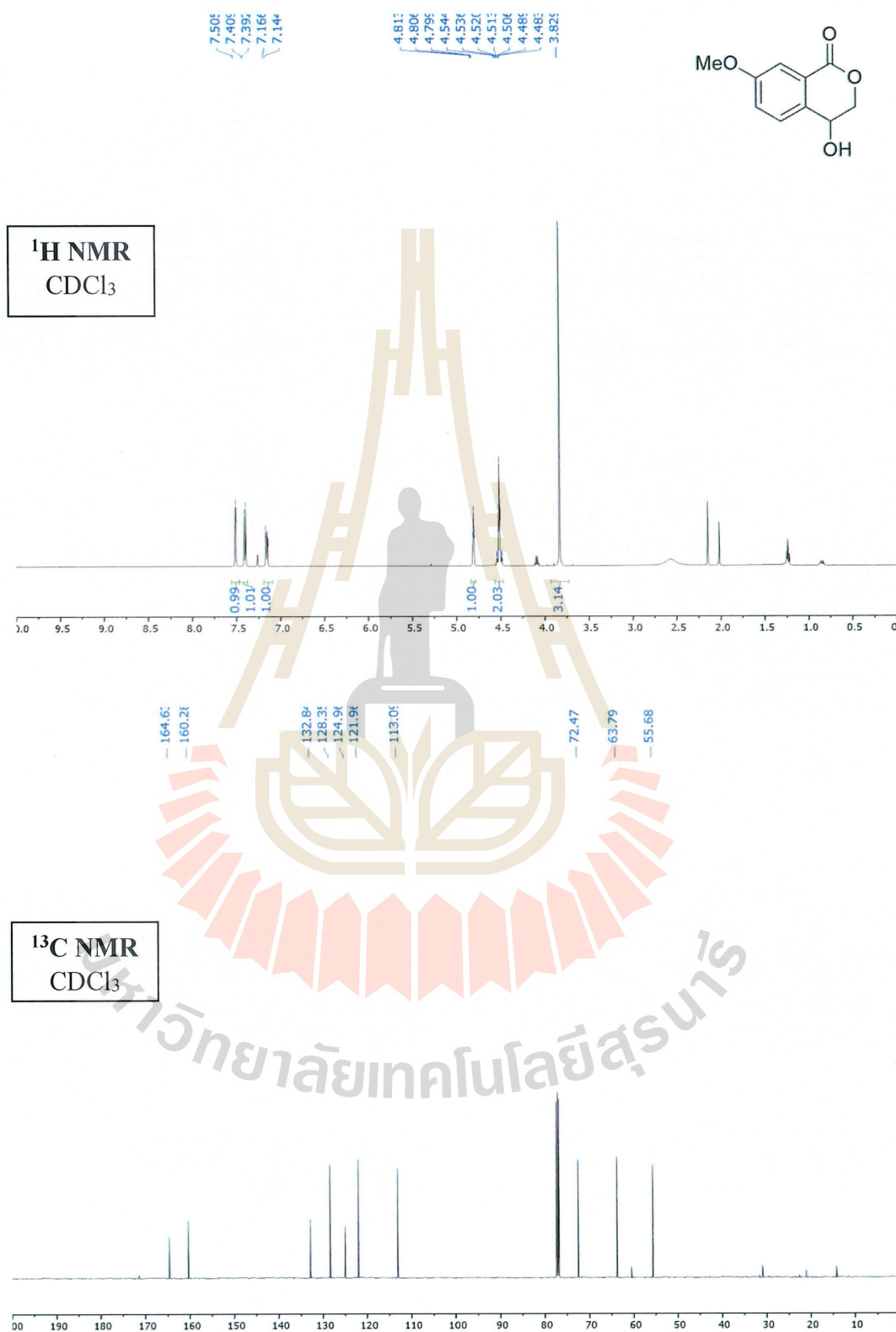
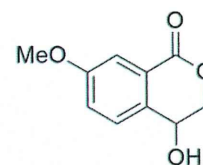
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CDCl₃



¹³C NMR
CDCl₃



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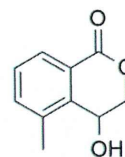


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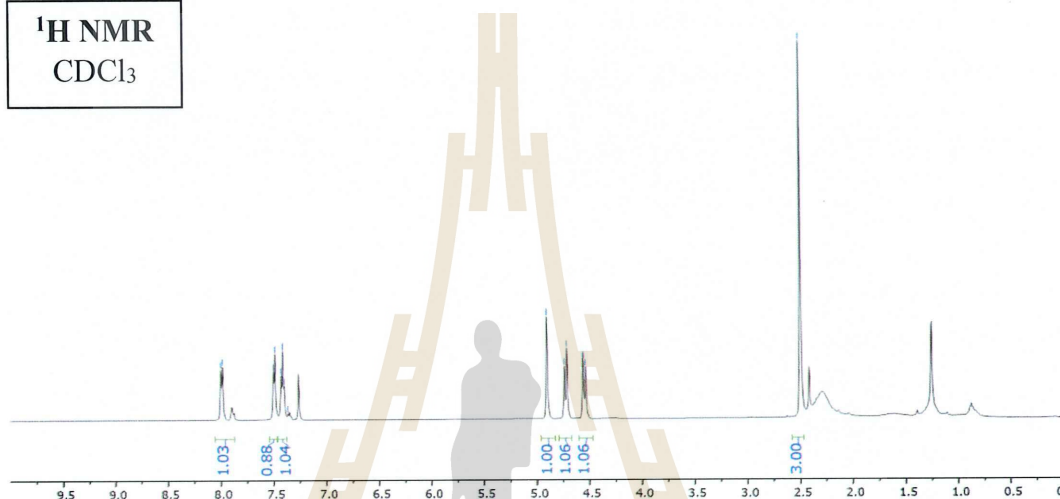
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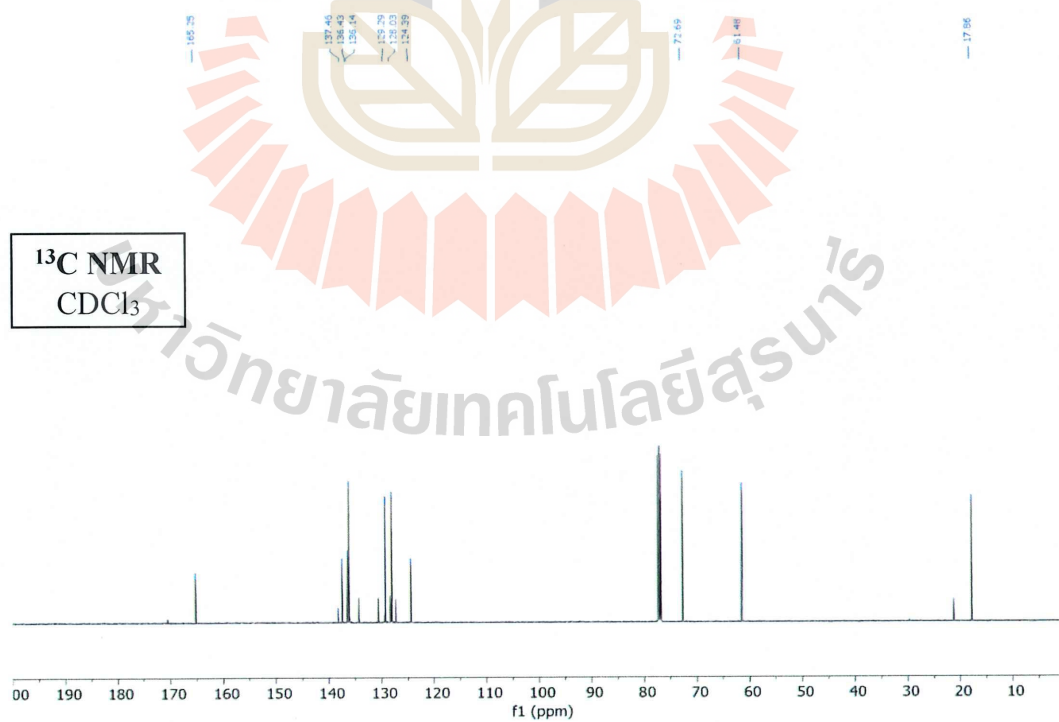
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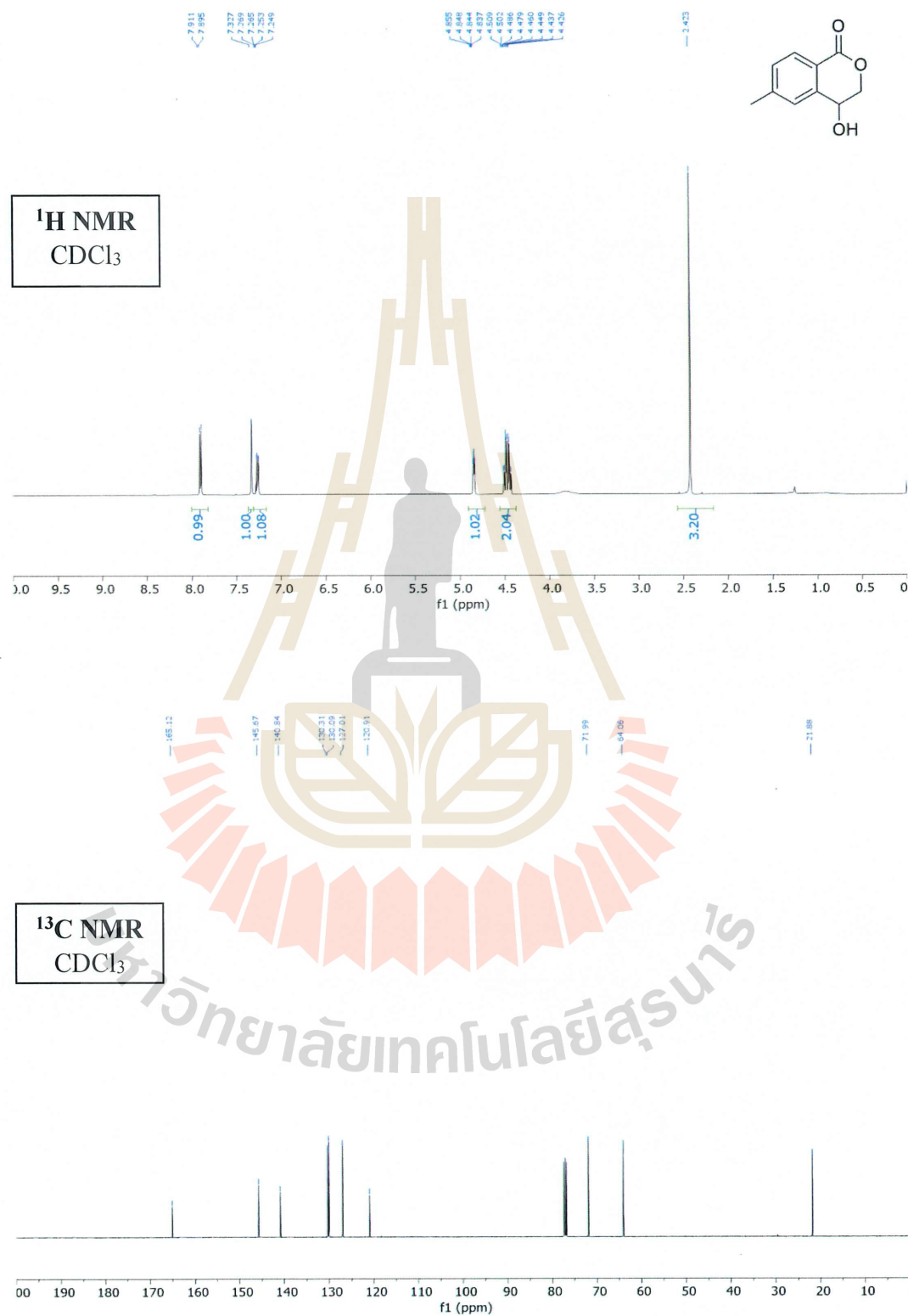
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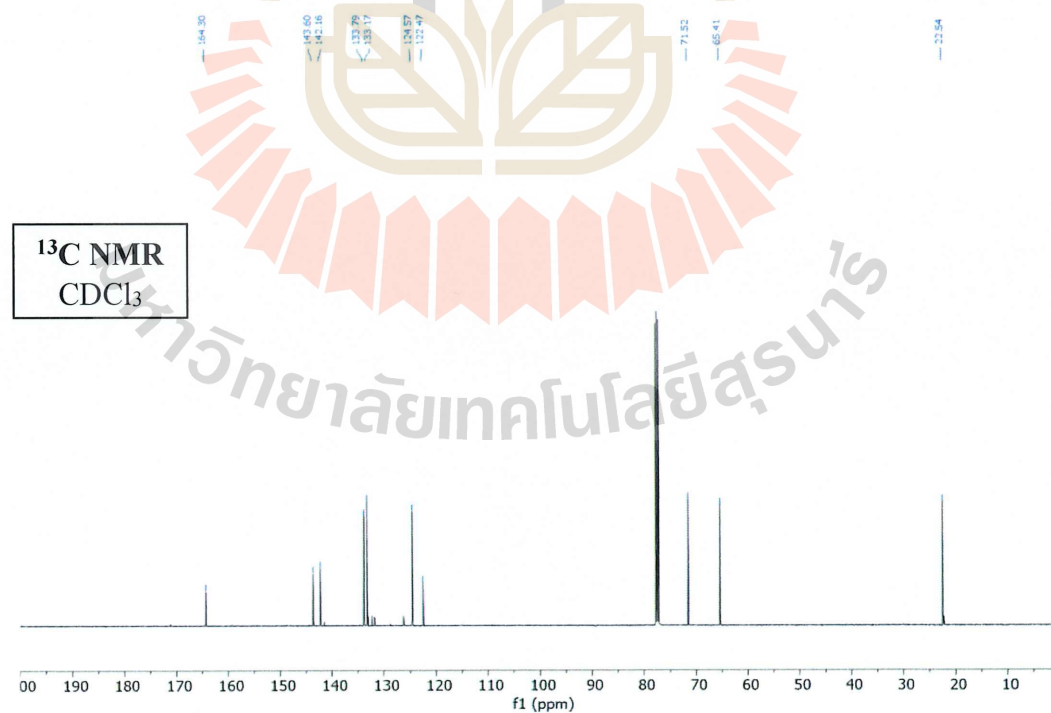
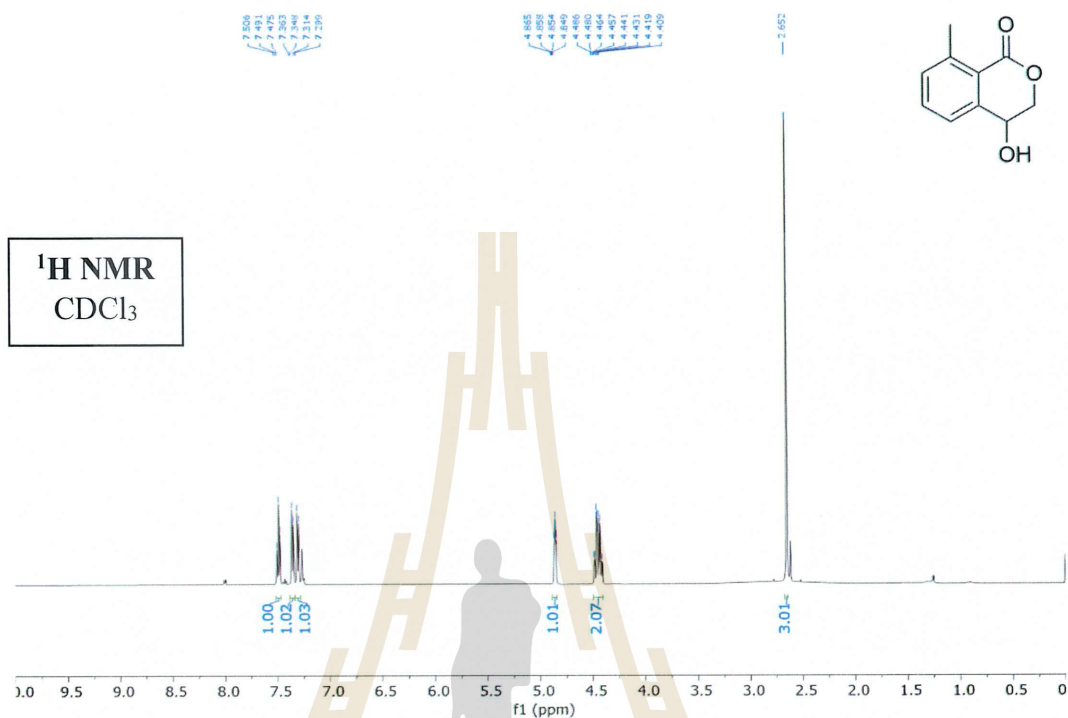
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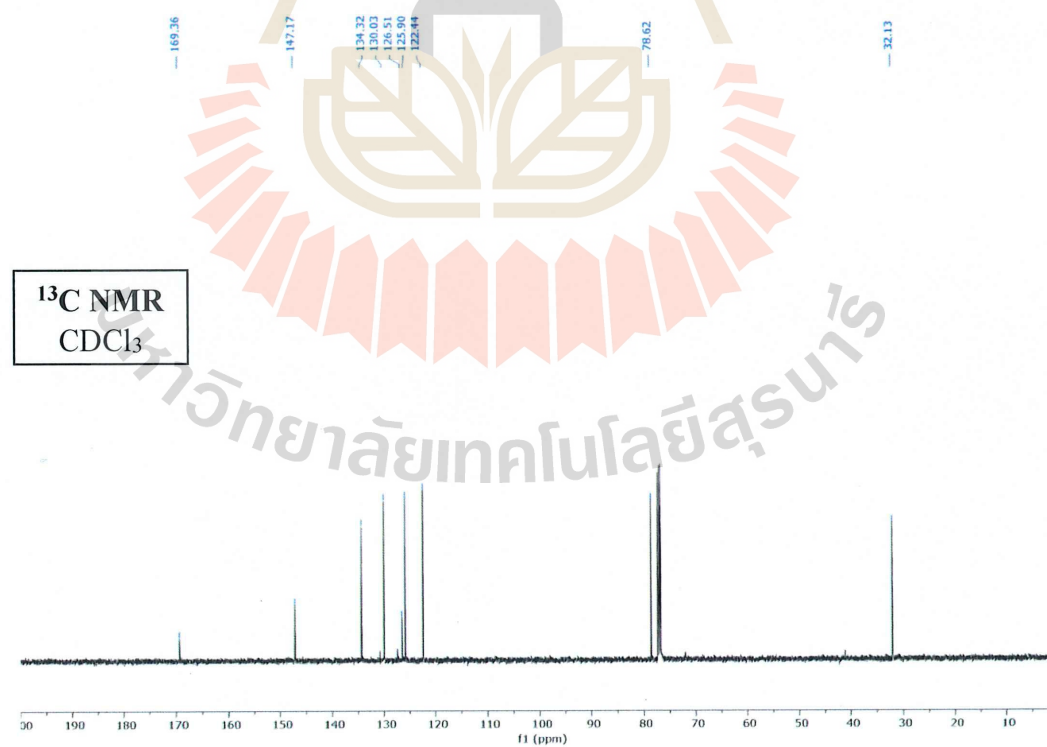
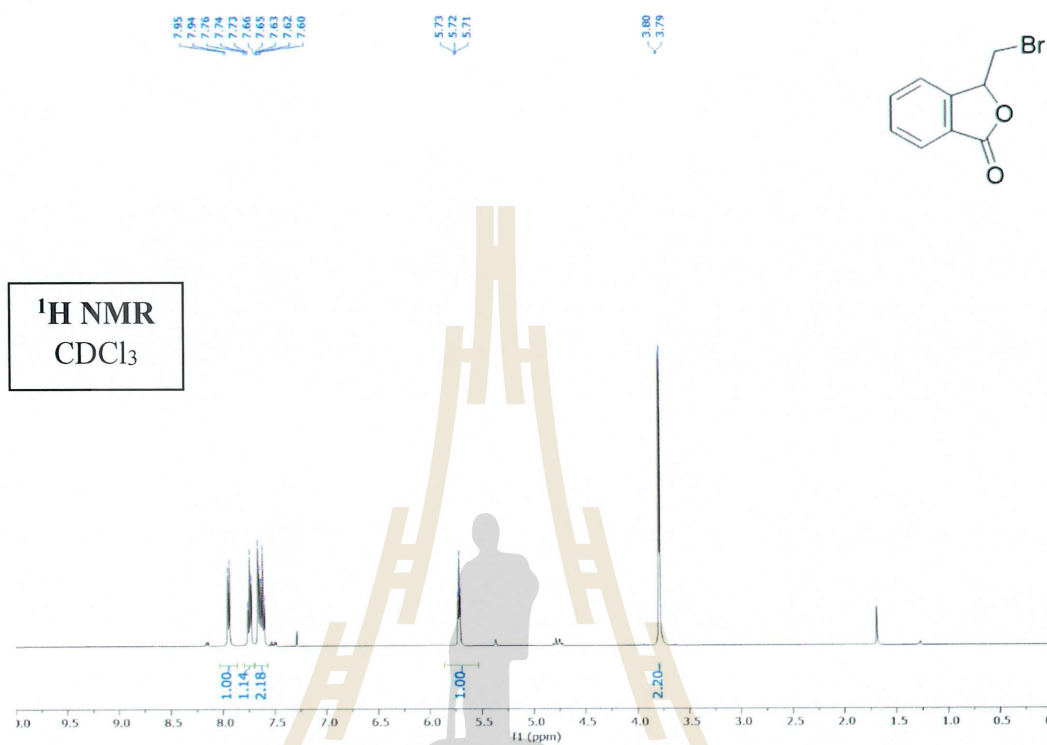
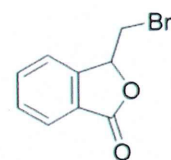
4-Hydroxy-6-methylisochroman-1-one (3d)



4-Hydroxy-8-methylisochroman-1-one (3e)



3-(Bromomethyl)-1(3H)-isobenzofuranone



APPENDIX B

PUBLICATION AND PRESENTATIONS

B.1 List of publications

Krongyut, C., Wiriya, N., Saiyasombat, W., Chansaenpak, K., Sripattanakul, S., Kamkaew, A., & Lai, R. Y. (2024). Chemoenzymatic Cyclization by Vanadium Chloroperoxidase for Synthesis of 4-Hydroxyisochroman-1-Ones. *ChemBioChem*, e202400697.

Huynh, T. N. T., Nguyen, K. T., Krongyut, C., Lai, R. Y., Sukwattanasinitt, M., & Wacharasindhu, S. (2025). Micelle-enabled bromination of α -oxo ketene dithioacetals: mild and scalable approach via enzymatic catalysis. *Organic & Biomolecular Chemistry*.

B.2 List of poster presentation

Chisanu Krongyut, Nittaya Wiriya, Anyanee Kamkaew, Rung-Yi Lai¹. (August 2024). Enzymatic Cyclization of 2-Vinylbenzoic Acid and Its Derivative. **The 18th International Symposium of the Protein Society of Thailand**, Bangkok, Thailand

มหาวิทยาลัยเทคโนโลยีสุรนารี



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Research Article
doi.org/10.1002/cbic.202400697



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Chemoenzymatic Cyclization by Vanadium Chloroperoxidase for Synthesis of 4-Hydroxyisochroman-1-Ones

Chisanu Krongyut^{*,[a]} Nittaya Wiriyat^{*,[a]} Worakrit Saiyasombat^{*,[a, b]} Kantapat Chansaenpak^{*,[c]} Sineenat Sripattanakul^{*,[a]} Anyanee Kamkaew^{*,[a]} and Rung-Yi Lai^{*,[a, d]}

4-Hydroxyisochroman-1-ones belong to the class of the secondary metabolite 3,4-dihydroisocoumarins. They exhibit a wide range of biological activities. These compounds can be synthesized through halocyclization using hypervalent iodine species or *N*-bromosuccinimide, followed by hydrolysis. Nonetheless, the reactions required specific conditions and generated toxic byproducts. In this study, *Curvularia inaequalis* vanadium chloroperoxidase (CVCPO) catalyzed the chemo-

enzymatic cyclization of 2-vinylbenzoic acids with different electron-donating groups (1a–1e) to produce good yields of 4-hydroxyisochroman-1-ones (3a–3e) by adding KBr and H₂O₂ in citrate buffer (pH 5). The reaction mixture contained 30% DMSO to improve substrate solubility without enzyme activity loss. The condition is more environmentally friendly than chemical methods. Therefore, it offers an alternative approach for synthesizing 4-hydroxyisochroman-1-ones.

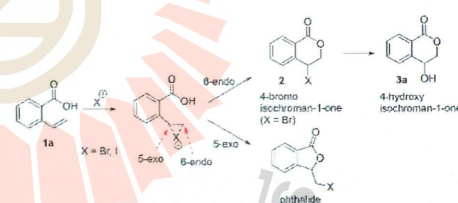
Introduction

3,4-Dihydroisocoumarin is an important skeleton of secondary metabolites.^[1] A derivative, 4-hydroxyisochroman-1-one, belongs to a family of polyketide metabolites isolated from bacteria and fungi (Figure 1). Studies indicated that 4-hydroxyisochroman-1-one derivatives exhibit a variety of biological activities, including cytotoxic, antifungal, and antibacterial properties.^[2–6] Therefore, synthesizing these compounds has garnered significant interest in developing different methodologies. Previously, the hetero-Diels–Alder cycloaddition of ortho-quinone dimethides followed by oxidation^[7] and the oxidative rearrangement of isobenzofurans followed by reduction^[8,9] were developed. However, it can be challenging to implement due to either substrate availability or reaction conditions.

Since 4-hydroxyisochroman-1-ones have a lactone structure, we reasoned that intramolecular cyclization of 2-vinylbenzoic acid **1a** is necessary for lactonization (Scheme 1). Therefore, the



Figure 1. Natural products containing the 4-hydroxyisochroman-1-one skeleton.



Scheme 1. Proposed 6-endo and 5-exo halocyclization of 2-vinylbenzoic acid **1a**. The 6-endo product **2** can be finally converted to 4-hydroxyisochroman-1-one **3a**.

key step would be the activation of alkene by electrophilic halogenation, followed by the nucleophilic addition of carboxylic acid via the 6-endo pathway for the formation of **2** instead of the 5-exo pathway for phthalide **3** formation.^[10]

In one study, a catalytic amount of the chiral hypervalent iodine (III) species, produced by the oxidation of chiral iodoarene, catalyzed the halocyclization of alkenylbenzoates, resulting in the enantioselective formation of acetylated 4-hydroxyisochroman-1-one derivatives.^[11] After the hydrolysis with K₂CO₃, 4-hydroxyisochroman-1-ones were produced. However, this approach has the drawback of requiring mCPBA and BF₃, a strong Lewis acid, for the cyclization reaction. In another

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report, a stoichiometric amount of *N*-bromosuccinimide (NBS) was used to catalyze the bromocyclization of styrene-type carboxylic acids, resulting in the formation of 4-bromo-3,4-dihydroisocoumarins **2**,^[14] which could subsequently be transformed into 4-hydroxyisochroman-1-one **3a** after a displacement reaction. Nonetheless, the cyclization process spanned several days to complete and required a stoichiometric NBS, resulting in the production of succinimide as a waste product. Additionally, both techniques necessitated low temperatures and anaerobic environments. Although the 6-endo products dominated in both scenarios, the 5-exo cyclization yielded noticeable amounts of side products.

Historically, hazardous and corrosive molecular halogens (X_2) was used for halogenations.^[13] Organic halogenating reagents like *N*-halosuccinimides have largely taken place of X_2 , although their production still requires elemental dihalogens. From an environmental perspective, one alternative approach is to use a mild oxidant, such as H_2O_2 , to oxidize a halide to the corresponding hypohalite, with the process being catalyzed by biocatalysts.^[14] Heme-dependent chloroperoxidase from *Caldariomyces fumago* (CFCPO)^[15,16] and vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (CVCPO)^[17–19] were reported to generate hypohalite for catalyzing the halocyclization of allenols or γ,δ -unsaturated carboxylic acids and alcohols. However, CFCPO is vulnerable to increased H_2O_2 concentrations, leading to oxidation and degradation of the porphyrin cofactor at the active site. Therefore, CVCPO has attracted increased interest due to its robust activity in conditions with elevated levels of H_2O_2 and organic solvents.^[20]

To gain a clearer insight into CVCPO's function in catalyzing this halocyclization of 2-vinylbenzoic acid **1a** (Scheme 1), we initially investigated this reaction with the addition of KBr and H_2O_2 as a model. Surprisingly, 4-hydroxyisochroman-1-one **3a** appeared as the main product, rather than 4-bromoisochroman-1-one **2**. The 5-exo cyclization product was not detected, unlike the chemical methods.^[10–12] However, 2-vinylbenzoic acid derivatives (**1b–1h**, Scheme 3) exhibited low solubility in the buffer. To enhance their solubility, several solvents were examined, and the buffer with 30% DMSO as a cosolvent was identified as the optimal condition for maximizing the reaction yield. Finally, the testing of optimal conditions for 2-vinylbenzoic acid derivatives **1b–1h** revealed that only the benzene ring containing electron-donating groups can be transformed into the corresponding products **3b–3e**.

Results and Discussion

In the 1990s, many mechanistic and molecular details of CVCPO were investigated.^[21–23] Furthermore, CVCPO was engineered via directed evolution to identify a triple mutant (P395D/L241V/T343A).^[24] In the standard monochlorodimedone (MCD) assay for CVCPO kinetic investigation, the variant showed bromination with a 6-fold increase and chlorination with a 2-fold increase at pH 5.0. However, this variant was never applied in a published biocatalytic reaction. We were curious about the

cause of this. Consequently, analyses were conducted to compare the CVCPO wild-type and the variant.

First, the wild-type and the variant of CVCPO with an *N*-terminal His-tag were individually overexpressed in *E. coli* BL21(DE3) and purified by Ni-NTA chromatography (Figure S1). No noticeable difference was observed in the protein yields. Subsequently, the purified CVCPO wild-type and the variant were subjected to the MCD assay to monitor the disappearance of MCD after reacting hypohalite at 290 nm using a UV-Vis spectroscopy. The results indicated that the variant oxidizes bromide and chloride at a faster rate compared to the wild-type (Figure 2). Their Michaelis–Menten kinetic parameters were determined by varying bromide concentrations at a fixed concentration of H_2O_2 (Figure S2). The k_{cat} of the CVCPO variant is 50.90 s^{-1} , which is about 5-fold larger than 10.86 s^{-1} in the wild-type. Considering that the activation of the alkene was proposed as the key step, we hypothesized that the variant could expedite the halocyclization process by generating hypohalite more rapidly. Consequently, the CVCPO variant will be used in the following study.

At the beginning of the study, the cyclization of 40 mM 2-vinylbenzoic acid **1a** was investigated (Table 1). We began by choosing the reported optimized condition of 160 mM KBr and 170 mM H_2O_2 as our starting point.^[25] When comparing the pH 5 condition (entry 4) to the other pH conditions (entries 1, 2, 3, 5, and 6), it was pleasing to observe a complete conversion after 24 hours. Our optimal pH for the cyclization is consistent

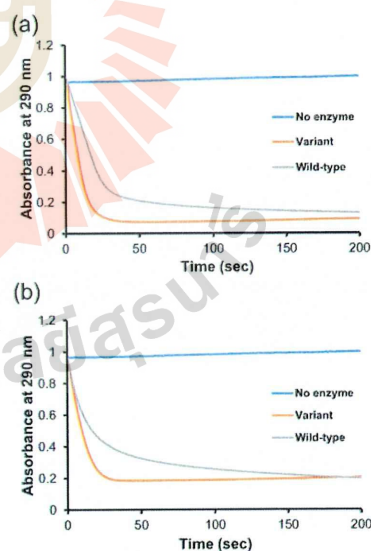


Figure 2. Activity comparison of CVCPO wild-type and the variant using the MCD assay. The assay of full reactions contained 1 mM H_2O_2 , 50 μ M MCD, 1 μ M enzyme, and 10 mM KCl (a) or KBr (b) in 100 mM citrate buffer (pH 5).

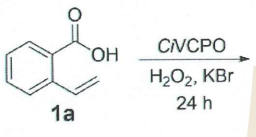
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Table 1. Optimization of the cyclization conditions of 2-vinylbenzoic acid 1a.

				
Entry	pH ^[a]	KBr (mM)	H ₂ O ₂ (mM)	Conversion ^[b] (%)
1	Water	160	170	12
2	3	160	170	25
3	4	160	170	30
4	5	160	170	Full
5	7	160	170	28
6	9	160	170	7
7	5	40	170	32
8	5	80	170	50
9	5	120	170	80
10	5	160	40	53
11	5	160	80	69
12	5	160	120	77
13 ^c	5	160	170	0
14	5	0	170	0
15	5	160	0	0

[a] Buffer used: acetate (pH 3), citrate (pH 4 and 5), and Tris-HCl (pH 7 and 9). [b] Determined by ¹H-NMR for the crude reaction mixtures. [c] The reaction did not contain CVCPO.

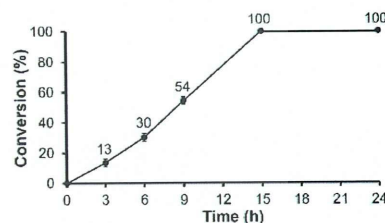


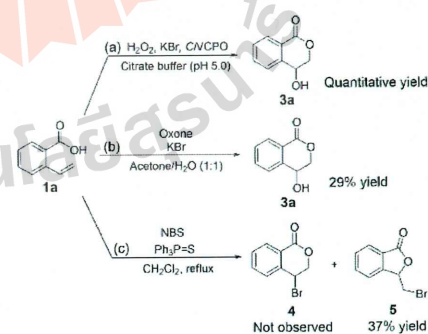
Figure 3. Time course of the chemoenzymatic cyclization of 2-vinylbenzoic acid 1a. The reaction contained 40 mM 1a, 160 mM KBr, 170 mM H₂O₂, and 400 nM CVCPO in 100 mM citrate buffer (pH 5). The conversion was calculated according to the 1a concentration by ¹H-NMR. Every time point was performed in triplicate.

reactions. Therefore, the reaction enantioselectivity was not investigated.

In our cyclization scenario (Table 1, Entry 4), CVCPO catalyzes the production of hypobromite through the oxidation of bromide using H₂O₂. To compare it with the chemical conditions generating electrophilic bromine, two reactions using commercially available reagents, oxone/KBr and NBS/triphenylphosphine sulfide, were tested for the cyclization of 1a (Scheme 2). Oxone containing potassium peroxymonosulfate has emerged as a green oxidant over the last decade for oxidizing halides to hypohalites for organic transformations.^[26] Therefore, in the cyclization of 1a, CVCPO and H₂O₂ were replaced by oxone in the cosolvents of acetone and water (Scheme 2b).^[27] However, ¹H-NMR analysis showed that the crude reaction mixture had only a 29% yield of product 3a. Additionally, we considered employing NBS to carry out halocyclization of 1a in the presence of triphenylphosphine sulfide (Scheme 2c). A similar reaction has been reported utilizing triphenylphosphine sulfide as a Lewis base catalyst to

with that of other CVCPO-catalyzed reactions.^[17–19,21,25] Conversions were determined by ¹H-NMR using *trans*-cinnamic acid as an internal standard. To reduce the amount of reagents used, various lower quantities of KBr and H₂O₂ were evaluated (entry 7–12); however, the reactions did not reach completion. Furthermore, the removal of the enzyme, KBr, or H₂O₂ from the reaction resulted in no conversion (entries 13–15). At various time intervals, samples from the reaction mixture were taken and subsequently extracted. The ¹H-NMR analyses indicated that the conversion steadily progressed to completion over 15 hours (Figure 3). The reaction time was set at 24 hours to ensure a complete reaction.

Following the completion of the reaction, the major product was identified by TLC and ¹H-NMR analysis of the crude mixture (Figure S3). Therefore, the major product was isolated and characterized by ¹H-NMR, ¹³C-NMR, and high-resolution mass spectroscopy. According to a similar reaction pattern by NBS,^[32] 4-bromoisochroman-1-one 2 was initially expected. Surprisingly, the isolated product was characterized as 4-hydroxyisochroman-1-one 3a, which hydroxyl group was further confirmed by Fourier-transform (FT-IR) infrared spectroscopy (Figure S4). The signals of 1711 and 3391 cm^{−1} correspond to the carbonyl and hydroxyl groups, respectively. In addition, CVCPO has been shown to release hypohalite from its active site to catalyze



Scheme 2. Cyclization of 2-vinylbenzoic acid 1a in different conditions for 24 h. (a) CVCPO-catalyzed reaction. (b) Oxone oxidized KBr to generate hypobromite, which promoted the cyclization. (c) NBS promoted the cyclization in the presence of Lewis base catalyst (Ph₃P=S).

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catalyze the cyclization of 5-phenyl-4-pentenoic acid, resulting in the selective formation of the 6-endo cyclization product (with an endo/exo selectivity of 91:1).^[10] After the reaction workup, the TLC analysis revealed a complex reaction mixture. In addition, the only 5-exo cyclization product was successfully isolated with a yield of 37%. As a result, both experiments highlighted the advantages of the CVCPO-catalyzed cyclization in yielding a single 6-endo product with excellent yield.

Next, to explore the reaction potential for different 4-hydroxyisochroman-1-one synthesis, 5-methoxy-2-vinylbenzoic acid **1b** was first synthesized.^[27] During the setup of this biocatalytic reaction, we noted that substrate **1b** did not fully dissolve in the citrate buffer, leading to a reaction yield of merely 14% (Figure 4). Consequently, the organic solvents utilized as

cosolvents in different CVCPO reactions, such as DMSO, DMF, EtOH, and MeOH,^[25,29,33] were added to the reaction mixture at a final concentration of 30% to improve substrate solubility. The results indicated that by adding 30% DMSO, the corresponding product **3b** can be produced with the highest yield of 82% compared to other cosolvents (Figure 4).

Therefore, the addition of 30% DMSO enhanced substrate solubility while maintaining enzyme activity. To explore the capability of our catalytic system, substrates with different substituents were synthesized and tested (Scheme 3). The reaction yields were determined using ¹H-NMR. The results indicated that substrates **1b–1e** containing electron-donating groups can be transformed into the corresponding 4-hydroxy-4-hydroxyisochroman-1-one derivatives **3b–3e**, which were finally isolated and characterized using ¹H-, ¹³C-NMR, and HRMS. Unfortunately, the substrates containing electron-withdrawing groups (**1f**, **1g**, and **1h**) failed to undergo cyclization. One possible explanation is that the electron density of the vinyl group is decreased by the inductive effect of electron-withdrawing groups, which hinders the electrophilic addition of hypobromite.^[25]

In our initial proposed reaction (Scheme 1), the brominated product **2** was anticipated rather than the hydroxyl product **3a**. In the substrate scope tests (Scheme 3), only the hydroxyl products (**3a–3e**) were isolated. Furthermore, the NMR study of the crude mixture utilized in the time course experiment revealed no detectable byproducts. A similar result was observed in the cyclization of 4-pentenoic acid, resulting in the formation of hydroxylactone and bromolactone.^[17] Therefore, a mechanism for the formation of the hydroxyl product **3a** from substrate **1a** is proposed in Scheme 4. Initially, CVCPO catalyzes the formation of hypobromite, leading to substrate **1a** undergoing an electrophilic addition reaction that produces the bromonium intermediate **6**. Subsequently, the intermediate **6** undergoes hydrolysis to generate vic-halohydrin **7**; the latter exists in equilibrium with the corresponding epoxide **8**, which is intramolecularly attacked by the carboxylate, resulting in the formation of the hydroxyl product **3a**.^[25]

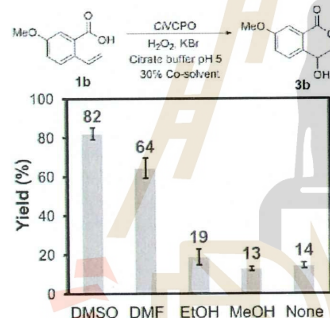
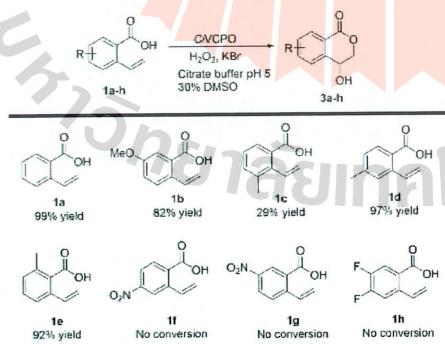
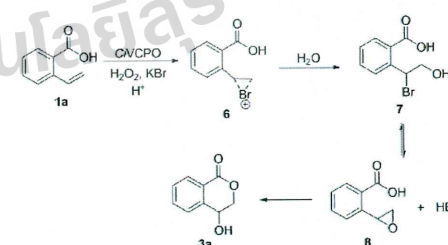


Figure 4. Cyclization of **1b** catalyzed by CVCPO with the addition of 30% co-solvent. General condition: 40 mM **1b**, 160 mM KBr, 170 mM H₂O₂, and 400 nM CVCPO in 100 mM citrate buffer containing 30% cosolvent. The yields of **3b** were determined by ¹H-NMR. Every solvent test was performed in triplicate.



Scheme 3. Substrate scope for CVCPO catalyzed cyclization of 2-vinylbenzoic acids (**1a–1h**). Reaction condition: 40 mM substrate, 160 mM KBr, 170 mM H₂O₂, and 400 nM CVCPO in 100 mM citrate buffer containing 30% DMSO for 24 h. The yields were determined by ¹H-NMR.



Scheme 4. Proposed mechanism for formation of the hydroxyl product **3a** from substrate **1a**.

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Conclusions

In summary, a chemoenzymatic cyclization of 2-vinylbenzoic acids was established to exclusively produce 4-hydroxyisochroman-1-ones through the 6-endo pathway. However, the substrate scope is limited to the ones containing electron-donating groups. To illustrate the advantages of this cyclization, two chemical methods were employed for the comparisons. The results indicated that CVCPO-catalyzed cyclization exceeds all expectations. Furthermore, this biocatalytic cyclization ensures that no toxic byproduct is generated. This breakthrough is not only impressive but also environmentally friendly.

Experimental Section

A detailed description of the enzyme preparation, purification, and kinetic determination, as well as a complete description of the substrate syntheses and all chemical characterizations, can be found in the Supporting Information.

Cyclization of 2-Vinylbenzoic Acid Substrates Catalyzed by CVCPO

Every reaction was conducted as follows: a substrate (40 mM) was added to 6 ml of citrate buffer (100 mM, pH 5.0) containing 30% DMSO in a 25 ml single-neck round-bottom flask. The mixture was stirred till the substrate dissolved. Then, H₂O₂ (170 mM), KBr (160 mM), and CVCPO (400 nM) were added to the substrate solution. The mixture was continued to stir at 600 rpm at room temperature for 24 hours. After the reaction, the pH of the solution was adjusted to 1–2 by adding 1 M HCl. The reaction mixture was then extracted with CH₂Cl₂ three times and dried over anhydrous Na₂SO₄. After filtration, the solvent was removed by a rotary evaporator. Finally, the product was purified using flash chromatography on silica gel. The purified product was characterized by ¹H NMR, ¹³C NMR, and HRMS. For the determination of yield and conversion, a known amount of *trans*-cinnamic acid as an internal standard was added into the reaction mixture before extraction. After that, the crude mixture was analyzed by ¹H NMR to determine the integration values of the substrate, the product, and the internal standard. Finally, the yield and conversion were calculated accordingly.

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

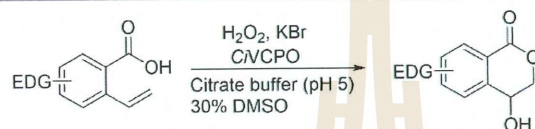
The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Vanadium chloroperoxidase · Isochroman-1-one · Biocatalyst · Halocyclization

- [1] A. Braca, A. Bader, N. De Tommasi, in *Studies in Natural Products Chemistry* (Ed.: Atta-ur-Rahman), Elsevier, 2012, ch. 7, pp. 191–215.
- [2] M. Isaka, A. Yangchun, S. Intamas, K. Kocharin, E. B. G. Jones, P. Kongsaree, S. Prabpai, *Tetrahedron* 2009, 65, 4396–4403.
- [3] R. Jansen, B. Kunze, H. Reichenbach, G. Höfle, *Eur. J. Org. Chem.* 2002, 2002, 917–921.
- [4] W. Zhang, K. Krohn, S. Draeger, B. Schulz, *J. Nat. Prod.* 2008, 71, 1078–1081.
- [5] S. Suzuki, T. Murayama, Y. Shiono, *Phytochemistry* 2005, 66, 2329–2333.
- [6] M. Taniguchi, M. Yanai, Y. Q. Xiao, T. Kido, K. Baba, *Phytochemistry* 1996, 42, 843–846.
- [7] M. F. Hentemann, J. G. Allen, S. J. Danishefsky, *Angew. Chem. Int. Ed.* 2000, 39, 1937–1940.
- [8] S. J. Hobson, A. Parkin, R. Marquez, *Org. Lett.* 2008, 10, 2813–2816.
- [9] B. A. Egan, M. Paradowski, L. H. Thomas, R. Marquez, *Org. Lett.* 2011, 13, 2086–2089.
- [10] S. E. Denmark, M. T. Burk, *Proc. Natl. Acad. Sci.* 2010, 107, 20655–20660.
- [11] M. Shimogaki, M. Fujita, T. Sugimura, *Eur. J. Org. Chem.* 2013, 2013, 7128–7138.
- [12] J. Chen, L. Zhou, C. K. Tan, Y.-Y. Yeung, *J. Org. Chem.* 2012, 77, 999–1009.
- [13] I. Saikia, A. J. Borah, P. Phukan, *Chem. Rev.* 2016, 116, 6837–7042.
- [14] B. O. Burek, S. Boimann, F. Hollmann, J. Z. Bloh, D. Holtmann, *Green Chem.* 2019, 21, 3232–3249.
- [15] J. Naapuri, J. D. Rolles, J. Keil, C. Manzana Sapu, J. Deska, *Green Chem.* 2017, 19, 447–452.
- [16] J. M. Naapuri, G. A. Åberg, J. M. Palomo, J. Deska, *ChemCatChem* 2021, 13, 763–769.
- [17] G. T. Höller, A. But, S. H. H. Younes, R. Wever, C. E. Paul, I. W. C. E. Arends, F. Hollmann, *ACS Sustain. Chem. Eng.* 2020, 8, 2602–2607.
- [18] J. M. Naapuri, P. K. Wagner, F. Hollmann, J. Deska, *ChemistryOpen* 2022, 11, e202100236.
- [19] S. H. H. Younes, F. Tieves, D. Lan, Y. Wang, P. Süß, H. Brundiek, R. Wever, F. Hollmann, *ChemSusChem* 2020, 13, 97–101.
- [20] R. Wever, R. Renirie, F. Hollmann, in *Vanadium Catalysis* (Eds: M. Sutaradhar, A. J. L. Pombeiro, J. A. L. da Silva), The Royal Society Of Chemistry, 2020, ch. 24, pp. 548–563.
- [21] J. W. P. M. van Schijndel, E. G. M. Vollenbroek, R. Wever, *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* 1993, 1161, 249–256.
- [22] A. Messerschmidt, L. Prade, R. Wever, *Biol. Chem.* 1997, 378, 309–316.
- [23] J. W. P. M. Van Schijndel, P. Barnett, J. Hoelze, E. G. M. Vollenbroek, R. Wever, *Eur. J. Biochem.* 1994, 225, 151–157.
- [24] Z. Hasan, R. Renirie, R. Kerkman, H. J. Ruijsenaars, A. F. Hartog, R. Wever, *J. Biol. Chem.* 2006, 281, 9738–9744.
- [25] J. J. Dong, E. Fernández-Fuero, J. Li, Z. Guo, R. Renirie, R. Wever, F. Hollmann, *Chem. Commun.* 2017, 53, 6207–6210.
- [26] S. Alvi, V. Jayant, R. Ali, *ChemistrySelect* 2022, 7, e202200704.
- [27] V. Rai, G. S. Sorabadi, M. B. Maddani, *New J. Chem.* 2021, 45, 1109–1113.
- [28] Y. Aoi, S. Takahashi, N. Chatani, *Org. Lett.* 2023, 25, 3266–3270.
- [29] C. E. Wells, L. P. T. Ramos, L. J. Harstad, L. Z. Hesselort, H. J. Lee, M. Sharma, K. F. Bieganski, *ACS Catal.* 2023, 13, 4622–4628.
- [30] H. Li, S. H. H. Younes, S. Chen, P. Duan, C. Cui, R. Wever, W. Zhang, F. Hollmann, *ACS Catal.* 2022, 12, 4554–4559.

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RESEARCH ARTICLE



Curvularia inaequalis vanadium chloroperoxidase (CVCPO) catalyzed the chemoenzymatic cyclization of 2-vinylbenzoic acids with different electron-donating groups to produce

good yields of 4-hydroxyisochroman-1-ones by adding KBr and H_2O_2 in citrate buffer (pH 5) containing 30% DMSO.

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Chemoenzymatic Cyclization by
Vanadium Chloroperoxidase for
Synthesis of 4-Hydroxyisochroman-
1-Ones

มหาวิทยาลัยเทคโนโลยีสุรนารี

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PP078

Enzymatic Cyclization of 2-Vinylbenzoic Acid and Its Derivative**Chisanu Krongyut¹, Nittaya Wiriya¹, Anyanee Kamkaew¹, Rung-Yi Lai^{1,*}**¹ School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

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ABSTRACT

3,4-Dihydroisocoumarins, a class of natural lactones, are known for their diverse structures and significant biological activities, including anti-inflammatory and antitumoral properties. These compounds are abundant in nature, sparking considerable interest in their synthesis due to their biological importance and structural diversity. Traditionally, the chemical synthesis of 3,4-dihydroisocoumarins relies on Lewis acids to catalyze the cyclization of 2-vinylbenzoic acid. However, this method involves toxic chemicals, organic solvents, and harsh reaction conditions. This project developed a biocatalytic approach using the recombinant enzyme haloperoxidase from the marine fungus *Curvularia inaequalis* (CiVCPO) to catalyze the cyclization reaction. This method activates the alkene through hypohalite generation by CiVCPO under mild conditions. Under optimized conditions in citrate buffer at pH 5, the reaction achieved a quantitative yield after 24 hours. The products were characterized using nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HRMS), and Fourier transform infrared spectroscopy (FT-IR). Encouraged by these promising preliminary results, 2-vinylbenzoic acid derivatives were also tested, successfully generating various 3,4-dihydroisocoumarins.

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CURRICULUM VITAE

Mr. Chisanu Krongyut was born on June 15th, 1999, in Ubon Ratchathani, Thailand. He earned his Bachelor of Science (B.Sc.) in Biology with second-class honors in 2022 from the Institute of Science, School of Biology, at Suranaree University of Technology, Thailand. During his undergraduate studies, he conducted senior research on the population genetic diversity of *Habenaria rhodocheila* under the supervision of Assoc. Prof. Dr. Noodeuan Muangsaen. In 2022, Mr. Krongyut pursued a Master's degree in Biochemistry and Biochemical Technology in the School of Chemistry at Suranaree University of Technology. His studies were supported by a scholarship from the Research and Development Support Fund (OROG), awarded to graduate students whose faculty members receive external research funding. His master's thesis, supervised by Asst. Prof. Dr. Rung-Yi Lai, focused on alkene activation using *Curvularia inaequalis* vanadium chloroperoxidase (CVCPO) as a biocatalyst. The thesis, titled "Chemoenzymatic Cyclization by Vanadium Chloroperoxidase for Synthesis of 4-Hydroxyisochroman-1-Ones," explored innovative approaches to organic synthesis. Mr. Krongyut's research has been recognized with a grant from the National Research and Innovation Information System (NRIIS, Thailand), highlighting his contributions to the field of biochemistry and enzymology. His academic journey reflects a strong commitment to scientific research and innovation.