

DISSERTATION

Dihydroxylation of Quinolines and Isoquinolines by Recombinant Whole-Cell Mediated Biotransformations

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines
Doktors der technischen Wissenschaften unter der Leitung von

**Univ.-Prof. Dipl.-Ing. Dr. techn.
Marko D. Mihovilovic**

E163

Institut für Angewandte Synthesechemie

Eingereicht an der Technischen Universität Wien
Fakultät für Technische Chemie

Von

M. Sc. Muhammad Farooq Zia

Matr. Nr. 0527608
Siegessplatz 1-3/1/9
1220 Wien

Wien, 4 September 2015

To Shazia Farooq Zia

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Acknowledgements

First and foremost, I thank Professor Marko D. Mihovilovic for his excellent supervision for the research reported in this thesis. He offered enormous motivation for my work, with a caring and patient approach, and facilitating an excellent atmosphere for my research. Without his inspirational guidance, his enthusiasm, his encouragements, and his unconditional help, I could have never finished my doctoral work. He was very approachable throughout the period, always there with open doors and a big smile for whatever problems I had to discuss with him. Needless to mention is also the fact that his immense knowledge was key motivation throughout my PhD – I cannot express enough gratitude for what it meant to me to be the member of his prestigious research group.

In the same vein, I would like to express my gratitude to the Professor Peter Stanetty who showed great interest in my research and was never short of useful advice and suggestions in the last few years. I also wish to express my sincere thanks to Dr. Michael Schnürch for proof reading the draft and for his valuable input towards finalizing the draft. I would take the opportunity to also thank Dr. Florian Rudroff and Dr. Thomas Fischer for their support as senior fellows in the bioorganic research division of our group – they were go to guys for any problems and would never sit easy until it was resolved.

I want to thank the Hungarian research collaborators for providing the starting materials for the screening. I am also grateful to Professor Christian Hametner for measuring various high resolution NMR spectra and his great contribution in interpreting the structures of new compounds. I would like to thank Professor Kurt Mereiter and Professor Matthias Weil for X-Ray structure analysis of new compounds. I also gratefully acknowledge the staff of the Department of Applied Synthetic Chemistry, who are many to name individually here, for giving me all necessary assistance during my studies.

I owe my gratitude to all those people who have made this dissertation possible and because of whom my doctoral experience has been one that I will cherish forever. In particular, I would like to thank my colleagues Johanna Hammerle, Maximillian Haider, Navid Dastbarwerdeh, Dianna Oski Richi, Radoslav Flasik, Dario Bianchi, Daniela Rial, Marko Susnik, Marko Spina, Michael Schön, Muhammad Abdelhamid, Abraham Garcia Zapeda, Ather Farooq Khan, Michael Fink, Saima Feroz, Thomas Wiesinger, Laurin Wimmer, Niko, and Gerit.

I would like to thank Florian Untersteiner for his deep knowledge and fruitful discussions and support for computer to keep the things running smoothly.

I gratefully acknowledge my friend Professor Asghar Zaidi (of University of Southampton, the UK) for offering thorough and excellent feedback on an earlier version of this thesis. I am extremely thankful and indebted to him for sharing expertise, and sincere and valuable guidance and encouragements extended to me.

I want to thank Professor Ulrich Jordis for his great moral support as a father figure during my PhD research. I want to thank him for keeping me motivated and encouraging me to finalize the thesis. I cannot thank him enough for his extended support.

I am thankful to the Higher Education Commission of Pakistan (HEC) for providing me the scholarship. I also thank the OeAD (Österreichische Austauschdienst) for taking care of all the administrative matters during my studies. I would also like to thank once again Professor Mihovilovic for the financial support towards my studies from time to time.

I would also like to thank Kamran Mumtaz, Ikhtlaq Bhai, for the support and all the time spent at Central Chemicals. I also thank Jahangir Bhai for mentoring and supporting me all the way to Austria and providing the moral support always.

I want to thank my family for their love and support. Thanks to my parents, brothers and sisters and their families.

Finally, and most importantly, I would like to thank my wife Shazia. Her support, encouragement, quiet patience and unwavering love were undeniably the bedrock upon which the past ten years of my life have been built. She has been my best friend and great companion, loved, supported, encouraged, entertained, and helped me get through this agonizing period in the most positive way. I would like to thank her for giving me three beautiful and cheerful kids, Navira, Umar and Ayaan.

Abstract

Nowadays, several dioxygenases with complementary substrate profiles have become available for synthetic applications by creation of efficient recombinant whole-cell expression systems. In particular with such delicate enzyme complexes, production of the required biocatalyst within its natural environment inside of an intact host cells (predominantly *Escherichia coli*) is highly advantageous, as this strategy allows the utilization of such catalytic entities within asymmetric synthesis with a minimum expertise in enzymology and also proteins from pathogenic organisms can be studied in a benign and safe environment. The most abundantly utilized type of aryl dioxygenases (2,3-dioxygenases) incorporates the additional oxygen functionalities in *ortho*- and *meta*-position to an existing substituent at the aromatic core exclusively in *cis*-configuration usually in very high optical purity.

There are three different prototype enzymes distinguished by the nature of the preferably converted aromatic ring system toluene (TDO, from *Pseudomonas putida* F39/D), naphthalene (NDO, from *Pseudomonas putida* 119), and biphenyl dioxygenases (BPDO, from *Sphingomonas yanoikuyae* B8/36). The biocatalyst originated from *Pseudomonas putida* and was utilized within recombinant whole-cells of *E. coli*. The whole cell mediated biotransformations were carried out with three different enzymes *E. coli* JM109 (pDTG601A) expressing TDO, *E. coli* JM109 (DE3)(pDTG141) and *E. coli* JM109 (PVL1343-PMS13) expressing NDO.

Within this contribution we have focused our attention to substituted quinolines and isoquinolines and the scope of the biotransformation of a series was explored. While such systems have been investigated in the past, the chosen substituents (Cl, Br, OMe) adjacent to the ring nitrogen atom represented labile functionalities, which led to subsequent hydrolytic processes and ultimately to complicated compound mixtures. Based on rich chemistry associated to carboxylic acid derivatives and nitrile groups and the hydrolytic stability of the corresponding quinoline and isoquinoline derivatives, we investigate a small collection of such compounds in dioxygenase mediated biooxygenations. The present study establishes that several bicyclic azaarenes are good substrates in the dioxygenase catalyzed reaction, giving *cis*-dihydrodiol derivatives. In case of alkyl substituents monohydroxylated products were also observed. Complete structural assignment of novel metabolites was carried out using NMR and diffraction techniques.

The regio- and stereo-selectivity of the *cis*-dihydroxylation was found to be in accordance with the literature. Regioselective *cis*-dihydroxylation of the carbocyclic and the heterocyclic rings in the quinolines and isoquinolines (5,6 and/or 7,8 bonds), occurred to give the corresponding *cis*-dihydrodiol metabolites. The *cis*-diol metabolites formed by biooxygenations were isolated, purified, characterized and absolute configuration established by using heavy halogen assisted crystallographic studies of corresponding 4-iodobenzoate esters. The structures and absolute configurations of metabolites have been determined by NMR analysis and correlation to the existing data for similar compounds.

It was discovered that trichloroacetyl chloride could be used to protect the *cis*-diols as 5-membered cyclic carbonates. The scope and limitation of trichloroacetyl chloride towards 5-membered cyclic carbonate synthesis was elaborated with selected reactions with different classes including catechol, aliphatic, and cyclic diols. Cyclic carbonate synthesis was not selective in case of aliphatic diols. The aliphatic diols gave both cyclic carbonates and diesters with trichloroacetyl chloride. The diester was the major product of reaction as compared to the carbonate. This indicates that aromatic *cis*-diols can be protected as 5-membered cyclic carbonates with trichloroacetyl chloride whereas the aliphatic diols are not completely converted to cyclic carbonates.

Keywords: biotransformation; naphthalene dioxygenase (NDO); toluene dioxygenase (TDO); chemoenzymatic synthesis; catalytic hydrogenation; desymmetrization; *cis*-dihydrodiols.

Deutsche Kurzfassung

Durch die Entwicklung von effizienten rekombinanten Ganz-Zell-Expressionssystemen stehen heutzutage mehrere Dioxygenasen mit komplementären Substratprofilen für Syntheseanwendungen zur Verfügung. Insbesondere bei derart fragilen Enzym-Komplexen ist die Produktion von Biokatalysatoren in ihrer natürlichen Umgebung innerhalb von intakten Wirtszellen (bevorzugt *Escherichia coli*) äußerst vorteilhaft, zumal dadurch der Einsatz dieser Katalysatoren für asymmetrische Synthese mittels eines Minimums an Erfahrung in Enzymologie ermöglicht wird; zudem können Proteine von pathogenen Organismen in einem sicheren Umfeld untersucht werden. Die verbreitetste Gruppe von Aryldioxygenasen (2,3-Dioxygenasen) ermöglicht den Einbau von Sauerstoff an ortho- und meta-Position eines substituierten Aromaten in strikter cis-Konfiguration und in hoher optischer Reinheit.

Drei Prototypen-Enzyme können anhand des bevorzugten aromatischen Ringsystems als Substrat unterschieden werden: Toluol- (TDO aus *Pseudomonas putida* F39/D), Naphthalin- (NDO aus *Pseudomonas putida* 119) und Biphenyl-Dioxygenasen (BPDO aus *Sphingomonas yanoikuyae* B8/36). Die Biokatalysatoren mit Ursprung aus Pseudomonaden wurden in Form von rekombinanten Ganz-Zell Expressionssystemen in *E. coli* untersucht. Ganz-Zell-Biotransformationen wurden mittels dreier *E. coli* Überexpressionssysteme durchgeführt: *E. coli* JM109 (pDTG601A) zur TDO-Produktion, *E. coli* JM109 (DE3)(pDTG141) und *E. coli* JM109 (PVL1343-PMS13) zur NDO-Produktion.

Im Rahmen der vorliegenden Arbeit wurden insbesondere Biotransformationen von Chinolinen und Isochinolinen untersucht. Während mit diesen heterocyclischen Systemen prinzipiell bereits Vorarbeiten existierten, wurden bislang insbesondere Substituenten (Cl, Br, OMe) in Nachbarschaft zum Heteroatom studiert, welche hydrolytisch instabil waren und somit zu komplexen Produktgemischen führten. Somit wurde das Hauptaugenmerk auf Carbonsäurederivate und Nitrile gelegt, welche hydrolysestabiler sind und somit eine reichhaltigere und besser planbare Folgechemie erlauben. Eine kleine Substratbibliothek derartiger Verbindungen wurde hinsichtlich Biooxygenierungen mit Dioxygenasen untersucht. Dabei konnten mehrere bicyclische Azaarene als gute Substrate für diesen Enzymtyp identifiziert und die entsprechenden cis-Dihydrodiol-Metaboliten isoliert werden. Im Falle von alkylsubstituierten Substraten wurden auch mono-hydroxylierte Produkte beobachtet.

Es erfolgte eine weitgehend vollständige Strukturaufklärung der neuen Metabolite mittels NMR und Röntgenbeugung.

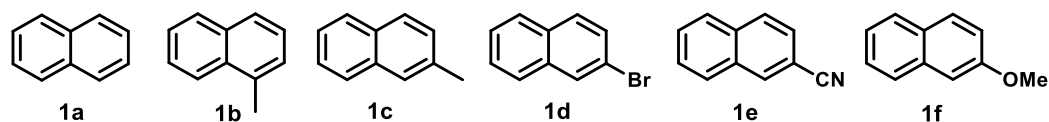
Die Regio- und Stereoselektivität der Dihydroxylierung war weitgehend analog zu Vorstudien in der Literatur. Regioselektive cis-Dihydroxylierung an carbocyclischen sowie heterocyclischen Systemen der Chinolin- und Isochinolinserie (5,6 und/oder 7,8 Bindungen) lieferten die cis-Dihydrodiol-metabolite in hoher optischer Reinheit. Die cis-Diolmetabolite wurden isoliert, aufgereinigt, spektral charakterisiert und schließlich hinsichtlich ihrer Absolutkonfiguration zugeordnet, wobei insbesondere der Schweratomeffekt mittels 4-Iodbenzoesäureesterderivate für Beugungsstudien herangezogen wurde. Die Strukturen und Konfigurationen der Metabolite wurden mittels NMR-Spektroskopie und Korrelationsexperimenten bestätigt und mit verwandten Verbindungen abgeglichen.

Im Zuge der Derivatisierungsstudien wurde Trichloracetylchlorid als neuartiges Schutzgruppenreagens für cis-Diole in Form von 5-gliedrigen cyclischen Carbonaten entdeckt. Der Anwendungsbereich dieser neuen Schutzgruppentechnik wurde bestimmt anhand strukturell unterschiedlicher Diole (Catechole, aliphatische und cyclische Diole). Dabei stellte sich die Umsetzung mit aliphatischen Diolen als nicht-selektive Reaktion heraus, wobei sowohl cyclische Carbonate wie auch Trichloractetyl-Diester gebildet wurden. Damit konnte unterschiedliche Reaktivität von Trichloracetylchlorid etabliert werden, wobei aromatische cis-Diole bevorzugt 5-gliedrige cyclische Carbonate bilden, wo hingegen aliphatische Diole vorzugsweise zu Diester reagieren.

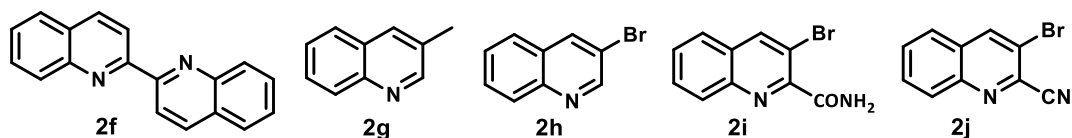
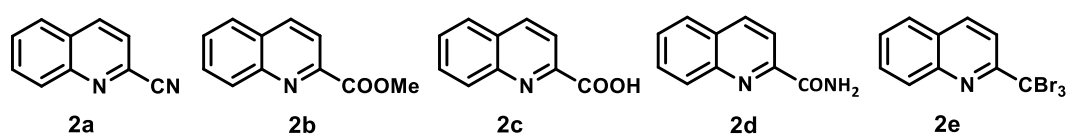
Schlüsselwörter: Biotransformationen; Naphthalin-Dioxygenase (NDO), Toluol-Dioxygenase (TDO); chemienzymatische Synthese; katalytische Hydrierung; Desymmetrisierung; *cis*-Dihydrodiole.

1 General schemes and substrate library

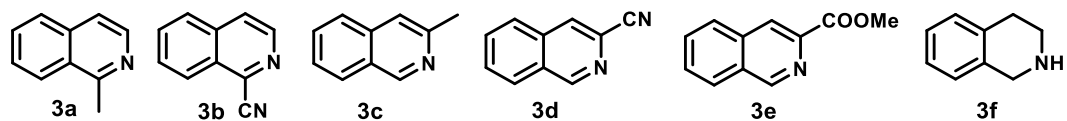
1.1 Substrate library



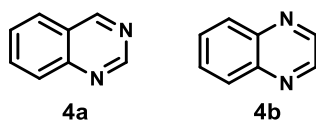
Naphthalenes



Quinolines



Isoquinolines

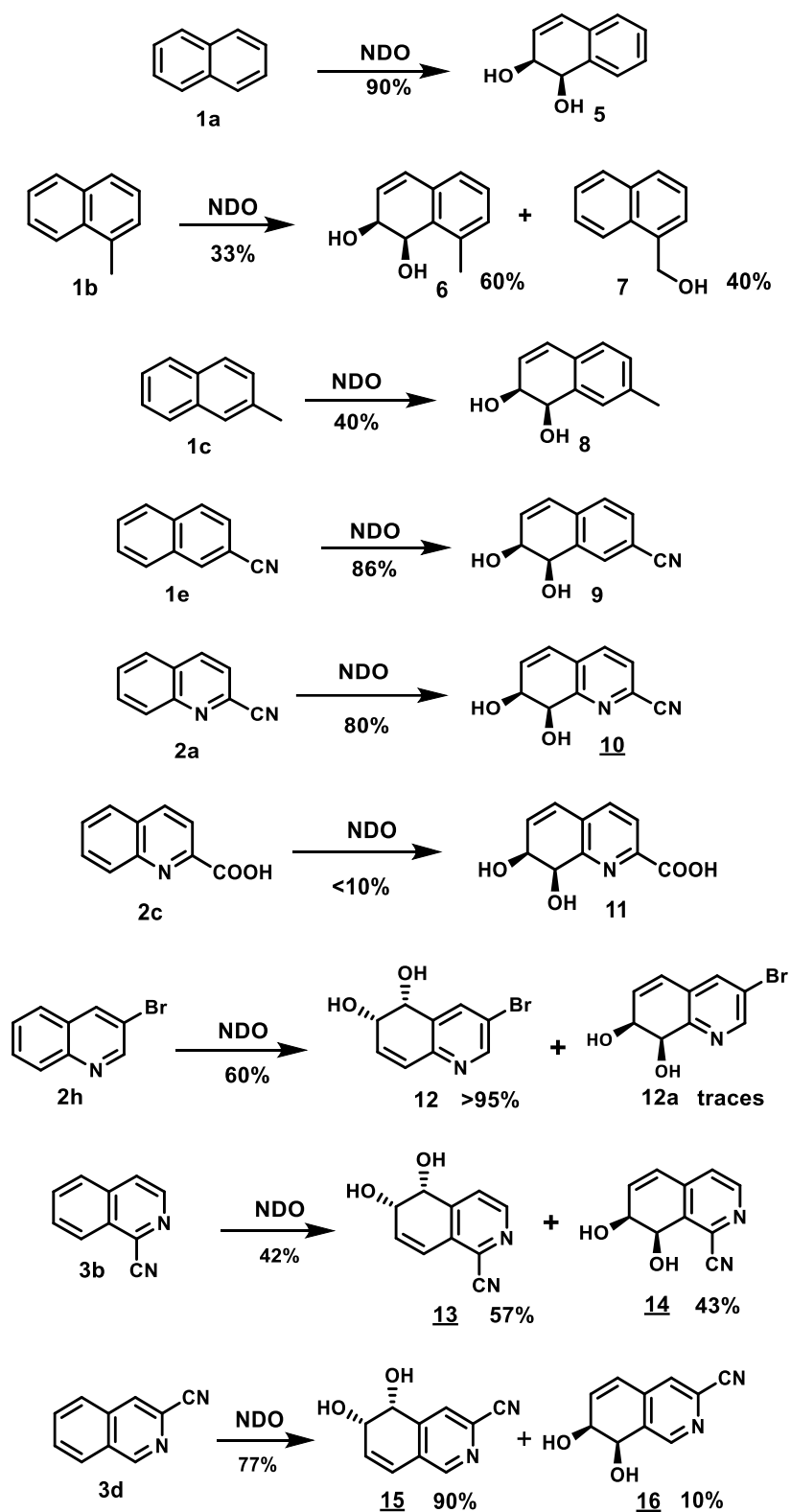


Quinazoline and quinoxaline

Scheme 1-1 Screening Substrates

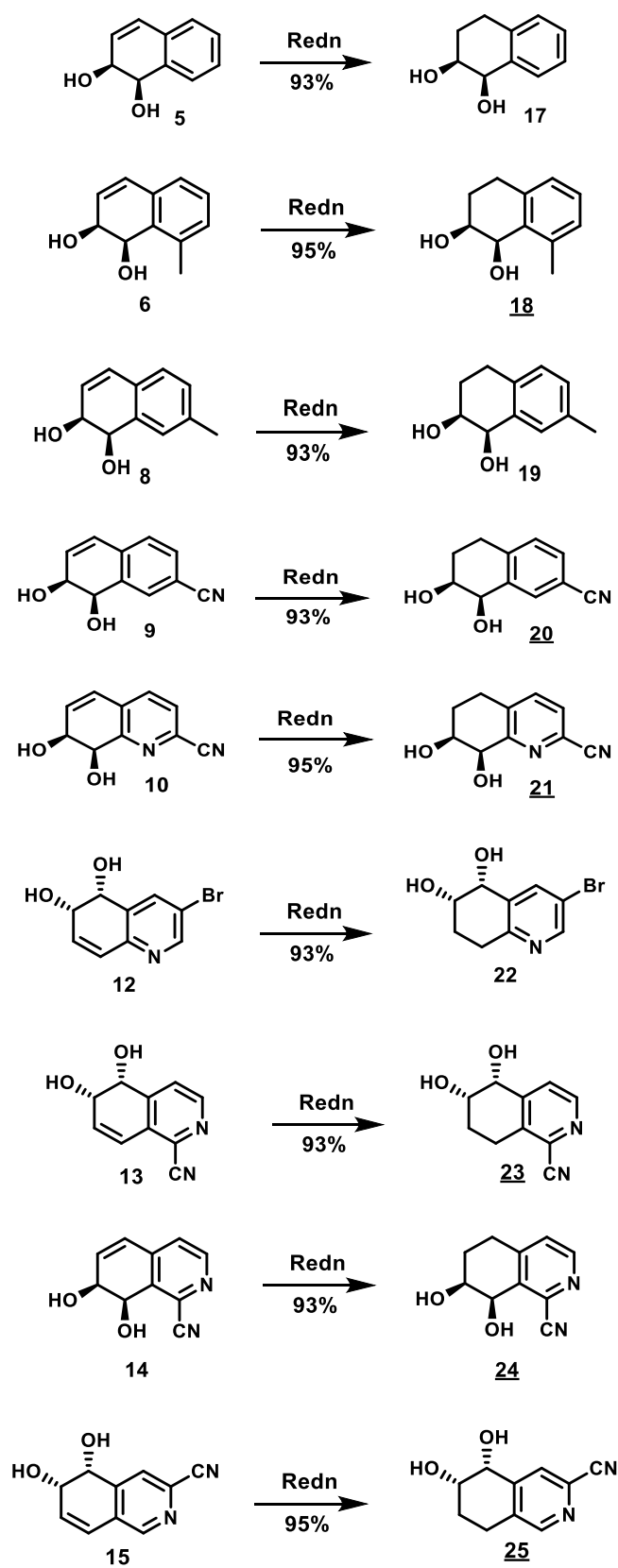
1.2 General Schemes

1.2.1 Synthesis of diols



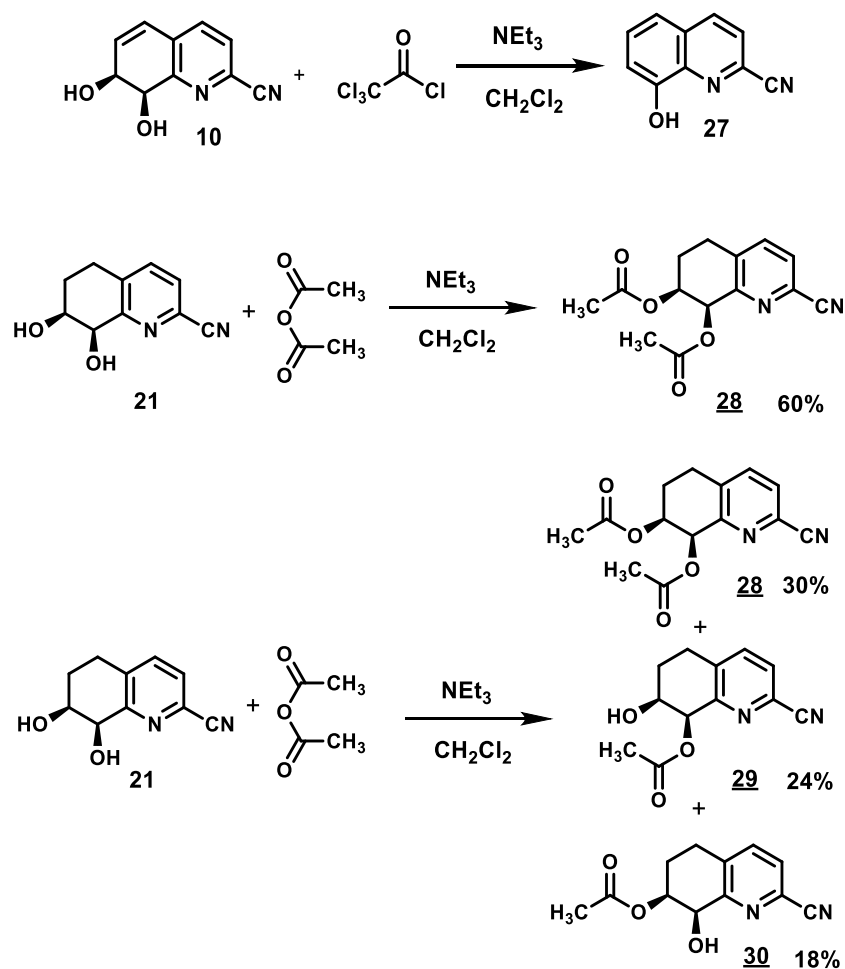
Scheme 1-2 Biotransformations

1.2.2 Reduction of diols



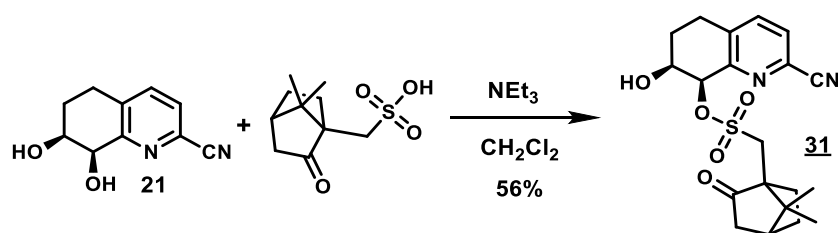
Scheme 1-3 Reduction of diols

1.2.3 Synthesis of acetate esters



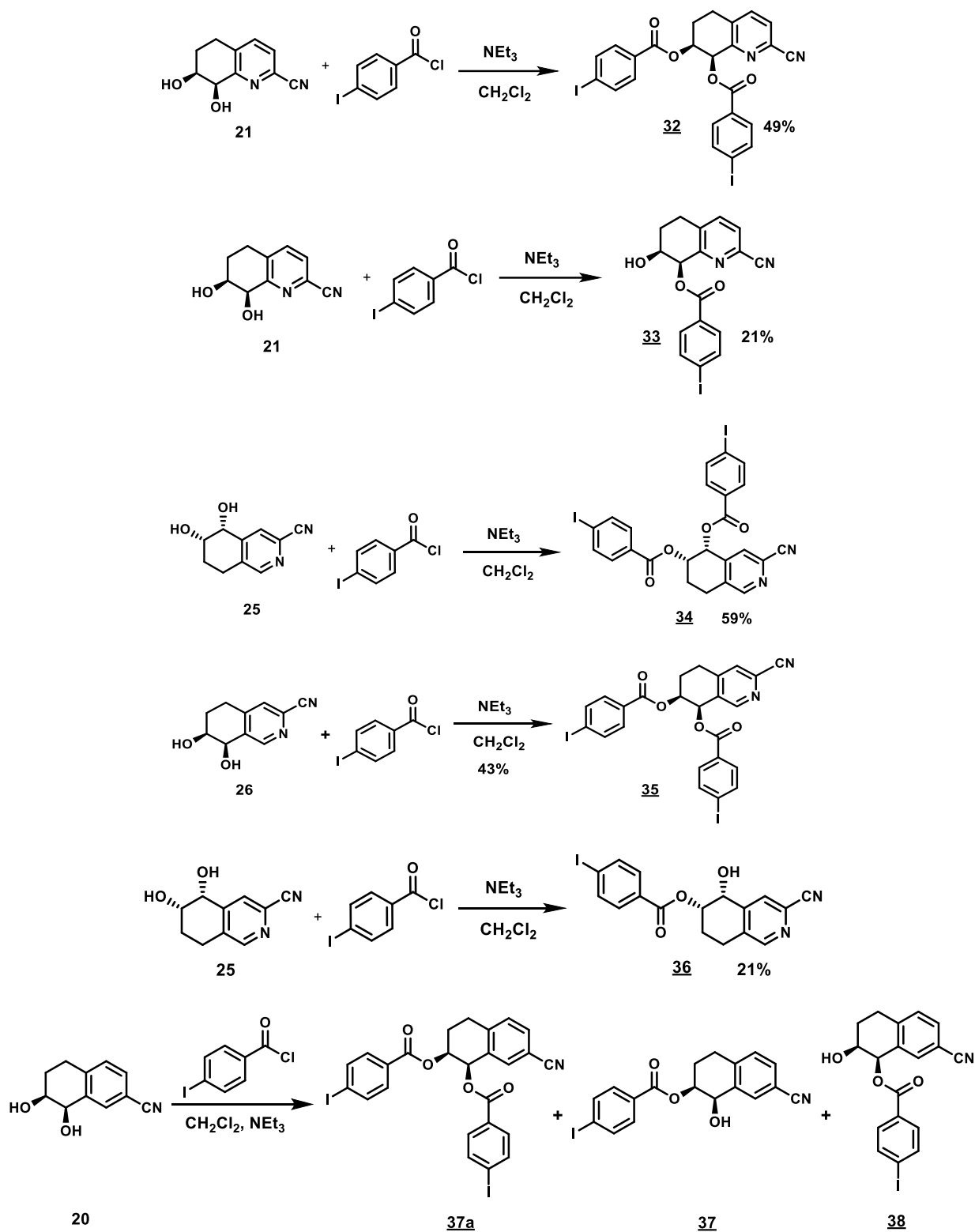
Scheme 1-4 Acetate ester synthesis

1.2.4 Synthesis of camphorsulfonyl ester

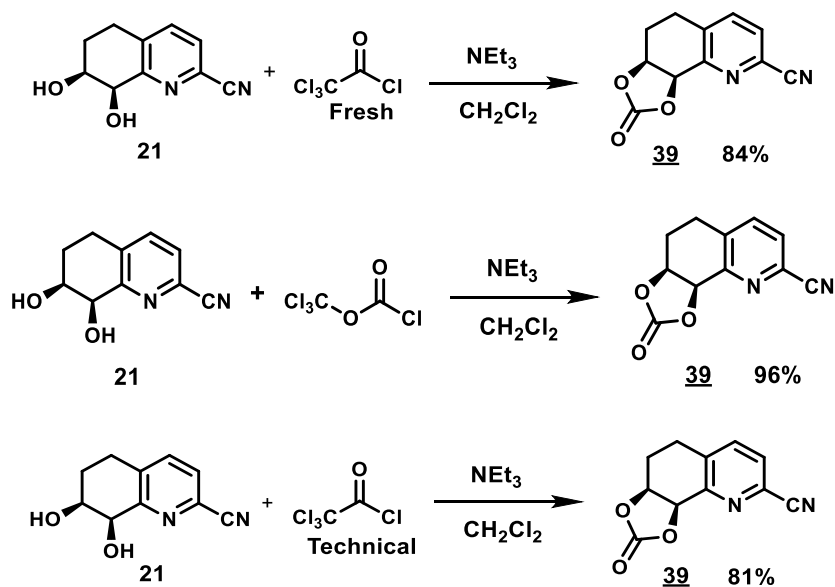
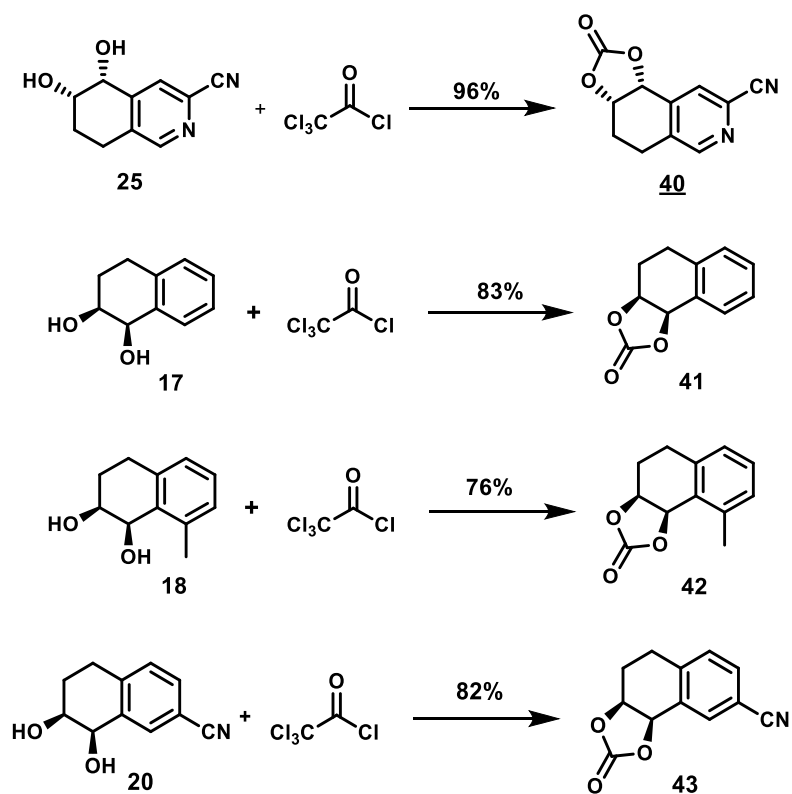


Scheme 1-5 Camphorsulfonyl ester synthesis

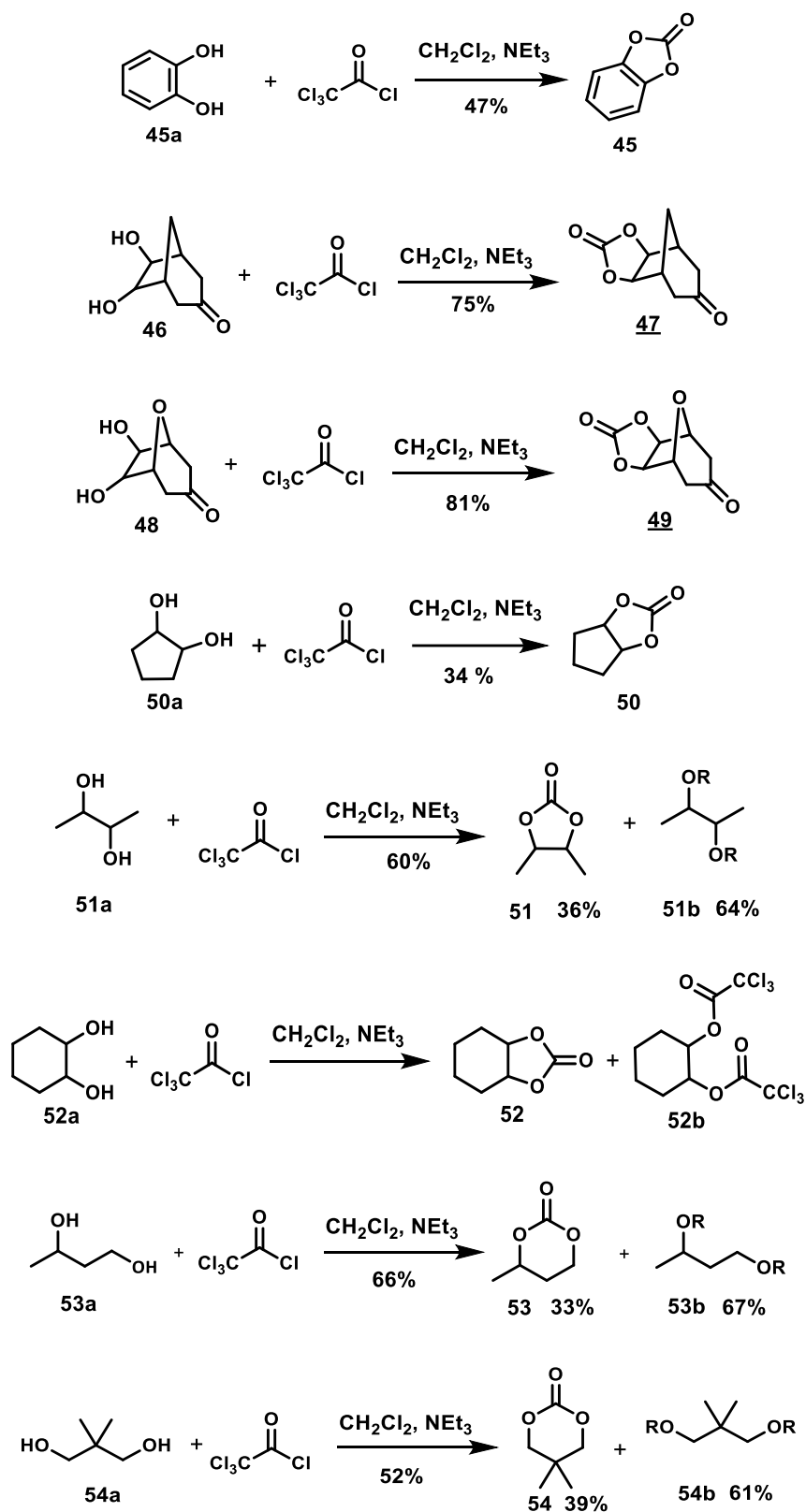
1.2.5 Synthesis of 4-Iodobenzoate esters



Scheme 1-6 Synthesis of 4-Iodobenzoate ester

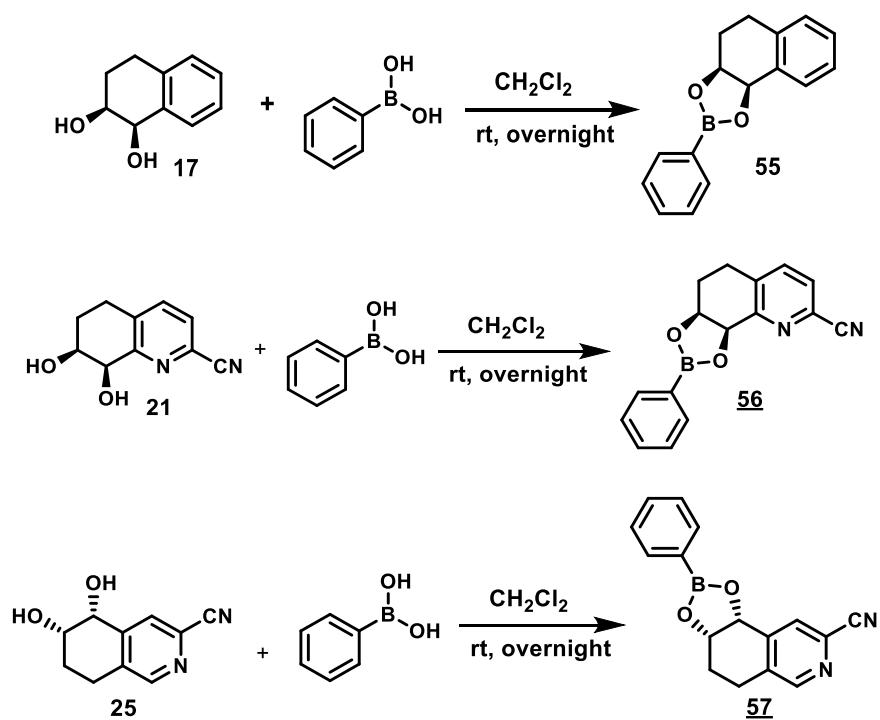
1.2.6 Synthesis of cyclic carbonates of *cis*-diolsScheme 1-7 Carbonate synthesis from *cis*-diol of quinoline-2-carbonitrileScheme 1-8 Synthesis of cyclic carbonates of *cis*-diols

1.2.7 Scope of acetyl chloride for cyclic carbonate synthesis



Scheme 1-9 Scope of acetyl chloride for carbonate formation

1.2.7.1 Synthesis of boronic esters



Scheme 1-10 Synthesis of boronic esters

2 Introduction

Chemical reactions can be catalyzed by inorganic, metal-assisted, organic, and biological means. Biocatalysis is about performing chemical reactions by means of biological systems, including isolated enzymes, whole cells or cell-free extracts. Whereas biotransformation can be defined as the reaction of chemical compounds in a living system, it does not need to be a process defined by the organism's natural metabolism. The terms seem to be interchangeable in the literature. For example, yeast reductions of keto esters, lipase-catalyzed acylations or hydrolyses would be classified as biocatalytic procedures. On the other hand, biotransformation is relatively random whole-cell fermentation of a compound yielding an isolable product. In such a case the organism would not utilize the substrate as carbon or energy source; otherwise no product could be isolated from the process.¹

2.1 Biodegradation, Biotransformation, and Biocatalysis (B3)

In order to exploit the natural metabolism of microorganisms and utilize them in chemistry, it is important to understand how they live energetically and how they transform in the environment. Biodegradation is the study of degradation of environmental pollutants by microorganism. Biotransformation and biocatalysis will deal with use of biodegradation knowledge about microorganism and using it to perform chemical reactions including industrial applications. In most cases, these interests overlap and better understanding of these help in designing sophisticated processes both on laboratory and industrial scale. In order to understand how biodegradation, biotransformation, and biocatalysis (B3) are related their interaction is explained in Fig 2-1.²

Polycyclic aromatic hydrocarbons (PAHs) and aza-polycyclic aromatic hydrocarbons (NPAHs) are formed during incomplete burning of coal, oil, gas, garbage, and are released from volcanoes, forest fires, vehicle exhausts, and other number of sources. They are highly carcinogenic due to their binding ability to the DNA and are toxic to the environment in a

¹ [1] T. Hudlicky and J. W. Reed, *Chem. Soc. Rev.* **2009**, 38, 3117-3132.

² [2] R. E. Parales, N. C. Bruce, A. Schmid and L. P. Wackett, *Appl. Environ. Microbiol.* **2002**, 68, 4699-4709.

number of ways.^{3,4} Microorganisms play an important role in degradation and detoxification of PAHs and NPAHs.^{5,6} Due to their stable conjugated structures it's difficult to decompose them by simple methods. Microorganisms including yeast, fungi and bacteria have the ability to grow in almost all kinds of environments and consuming whatever is available as energy source by natural adaptation by mutations.⁷

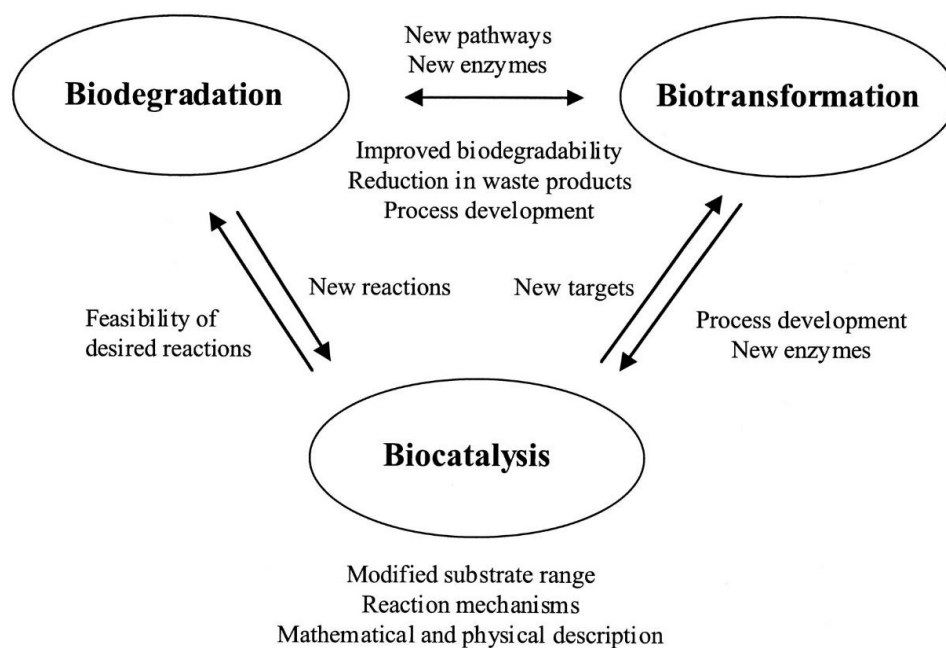


Fig. 2-1 Interdependence of three main application areas of enzyme catalysis

Microorganisms consume PAHs and NPAHs as carbon-sources during metabolic processes of degradation.⁸ They use molecular oxygen and metabolite toxic non-polar PAHs into polar hydroxy and epoxy derivatives. Epoxides are the major intermediates in the oxidative metabolism of aromatic double bonds. Such epoxides are reactive and enzymatically metabolized to other compounds such as dihydrodiols and phenols. Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and

³ [3] J.-S. Seo, Y.-S. Keum and Q. Li, *International Journal of Environmental Research and Public Health* **2009**, 6, 278-309.

⁴ [4] Y. Jouanneau, F. Martin, S. Krivobok and J. C. Willison in *Ring-hydroxylating dioxygenases involved in PAH biodegradation: structure, function and biodiversity*, Vol. Microbial Bioremediation of Non-Metals, Caister Academic Press, **2011**, pp. 149-175.

⁵ [5] G. Fuchs, M. Boll and J. Heider, *Nat. Rev. Microbiol.* **2011**, 9, 803-816.

⁶ [6] S. Fetzner, B. Tshisuaka, F. Lingens, R. Kappl and J. Huttermann, *Angewandte Chemie, International Edition* **1998**, 37, 576-597.

⁷ [7] D. Daubaras and A. M. Chakrabarty in *The environment, microbes and bioremediation: microbial activities modulated by the environment*, Vol. (Ed. E. Rosenberg), Microorganisms to Combat Pollution, Springer Netherlands, **1993**, pp. 1-11.

⁸ [8] C. Cerniglia in *Biodegradation of polycyclic aromatic hydrocarbons*, Vol. (Ed. E. Rosenberg), Microorganisms to Combat Pollution, Springer Netherlands, **1993**, pp. 227-244.

biodegradability.⁹ Different pathways are used by microorganisms in the degradation process of PAHs and NPAHs.^{10,11,12} During aerobic degradation of PAHs, microorganisms use enzymes, called oxygenases, which utilize the molecular oxygen for the oxidative attack.¹³

2.1.1 Enantioselective synthesis employing chiral catalysis

Enantioselective synthesis represents the chemical transformation of a compound that favors the formation of a specific enantiomer or diastereomer. As different enantiomers or diastereomers of a molecule often have different biological activity, it is very important in modern synthetic chemistry to explore processes that favor stereospecific compound preparation. This is very important in the pharmaceutical industry and also by other applications, including agricultural chemicals, flavors, fragrances, and materials. Most of the modern asymmetric drugs are single enantiomers. Asymmetric synthesis is generally achieved by either chiral auxiliaries or by metal catalysis by the introduction of ligands. Sometimes the catalysts are not general and for a particular reaction a large number of experiments is required in order to optimize the conditions and yields. Mostly chiral auxiliaries are required in stoichiometric amounts and unless they are cheap and easily recoverable it is very difficult to make it cost effective. Asymmetric catalysis has significant advantage over other synthesis processes as each molecule of catalyst, if continually regenerated, can yield many molecules of chiral product. Enantiomerically pure compounds are produced in nature with excellence by the transfer of chirality from enzymes. Chiral building blocks can be synthesized by applying the chemo-enzymatic approaches resulting in high enantiomeric purity by a biocatalytic reaction.

2.1.2 Chemical reactions vs biocatalysis

Oxidation reactions are very important in synthetic chemistry. Traditional methods for oxidation have limitations and drawbacks. There are often highly toxic heavy metal catalysts¹⁴ used for this reaction and one has to keep in mind the environmental and health aspect. There are most of the times undesired side reactions and overoxidations of substrates. Traditional

⁹ [9] R. Meulenberg, H. H. Rijnaarts, H. J. Doddema and J. A. Field, *FEMS Microbiol Lett* **1997**, *152*, 45-49.

¹⁰ [10] R. E. Parales, J. V. Parales, D. A. Pelletier and J. L. Ditty, *Adv. Appl. Microbiol.* **2008**, *64*, 1-73.

¹¹ [11] R. E. Parales and S. M. Resnick, *Soil Biol.* **2004**, *2*, 175-195.

¹² [12] R. E. Parales and J. D. Haddock, *Curr. Opin. Biotechnol.* **2004**, *15*, 374-379.

¹³ [13] K. Drauz, H. Gröger and O. May, *Enzyme Catalysis in Organic Synthesis, Third Edition*, Wiley-VCH, **2012**, p.

¹⁴ [14] R. Farrauto in *Industrial Catalysis: A Practical Guide, Vol.* (Ed. J. A. Kent), Handbook of Industrial Chemistry and Biotechnology, Springer US, **2012**, pp. 201-230.

organic reactions have less functional group tolerance. The application of highly reactive reagents on most of organic molecules creates problems of chemoselectivity due to similarity of functional groups. Regioselectivity and stereoselectivity are also significant challenges for classical oxidations. Development of selective catalyst for a particular reaction is not a trivial task. The substrate or the reagent needs to be adjusted in such a way that it does not undergo further reaction under same conditions. This leads us to perform lots of protection and deprotection steps for neighboring groups and synthesis of more complicated catalysts. Slight changes in the functional groups of substrate often cause significant effect on the efficiency of catalysts. Catalysts used are generally expensive and there is also economic aspect to be considered. Enzymatic transformation plays a key role for the processes where corresponding chemical transformation would be difficult, or non-selective via traditional methods.

In pharmaceutical industry many drugs are difficult to synthesize requiring multi-step reactions, large amounts of solvents, extensive purification steps, and low overall yields. Using enzymes as catalysts can improve the purity of the intermediates or end products and reduce the amount of organic solvents needed, resulting in a much greener overall manufacturing process. A good example of the impact of biocatalysis on pharmaceutical manufacturing is the production of pregabalin, the active ingredient in its \$3 billion per year drug Lyrica. Pfizer replaced a classical chemical processing step with a more efficient enzymatic step. The result was a 90% reduction in solvent used and a 50% reduction in starting material required. The E-factor of the process was reduced 5 folds from 86 to 17.¹⁵

2.1.3 Sources of biocatalysis

Biocatalytic reactions have highly diverse spectrum of sources ranging from humans to microorganisms. Microorganisms are rich sources of enzymes and the use of microorganisms in the field of biocatalysis has been vastly reported. The exploitation of microbial diversity in the quest for new enzymes with novel activities is one of the major research goals in biocatalysis. The use of recombinant microorganisms has been successfully applied to the synthetic methods meeting the challenging requirements.

¹⁵ [15] C. A. Martinez, S. Hu, Y. Dumond, J. Tao, P. Kelleher and L. Tully, *Organic Process Research & Development* **2008**, *12*, 392-398.

2.2 Methods used for biocatalysis

There are a number of factors including type of reaction, cofactor regeneration or recycling, scale of biotransformation, nature of substrate or product and nature of enzyme which determine the mode of biocatalysis. After a biocatalyst for a desired substrate has been identified, in order to convert it to a successful tool for that particular reaction, it is often required to clone, overexpress and genetically improve the biocatalyst. Biocatalysis can be carried out by using isolated enzymes, whole cells, resting and immobilized forms. Enzymes can be used as wild type without any genetic modification for a specific transformation. They can also be engineered to the desired degree of selectivity and nature of transformation.¹⁶

2.2.1 Isolated enzymes

Isolated enzymes provide better reaction control and often perform the reaction to ideal yields because there are no competing side reactions due to other enzymes that may occur when performed in the microorganism. Isolated enzymes are used in any suitable form dissolved in aqueous medium, suspended in organic solvents, immobilized or even under challenging conditions. Isolated enzymes can be used with relatively easy and simple procedure, apparatus, and workup after completion of reaction is also not tedious. They exhibit higher productivity due to high enzyme activities and higher concentration tolerance for both substrates and products. Sometimes, they are also easier to recover and can be reused.

Commonly existing problem of using isolated enzymes is their low stability. As enzymes are bound to their natural cofactors, it's necessary to setup reactions with cofactor recycling. Stabilities are limited as enzymes are exposed to their non-natural environment. Enzymes express reduced activities in organic solvents and loss of activity due to immobilization. Lots of efforts need to be done to handle cofactor regeneration problem. Addition of ancillary substrate or addition of another enzyme is some of the options to choose from.

2.2.2 Whole cell mediated biotransformations

For the transformations where single or multiple cofactors are required, whole cell mediated biotransformations are preferred. Also, if enzymes are too complex as is the case of oxido-reductases and unstable in isolated form, whole-cell biotransformations are preferred

¹⁶ [16] J. M. Woodley, *Biochem. Soc. Trans.* **2006**, *34*, 301-303.

method of choice. Whole cell mediated biotransformations can be performed by using both wild type and recombinant whole cells. Recombinant organisms are engineered in such a way that manufacture of desired protein and desired cofactors becomes the sole function of organism by the modification in its code and use of alternate carbon source. The cofactor regeneration of NAD(P)H takes place intracellularly, driven by the addition of a carbon source. Whole cells can be used as growing culture, as resting cells and can also be immobilized. They express higher activities due to natural environment. No cofactor recycling is necessary and no enzyme purification is required. Resting cells display higher activities and workup is also easy.

There are also limitations of using whole cells and there might be present other enzymes in the organism that can metabolize the substrate or the artificial metabolite towards unwanted products, ultimately decreasing yields. Other limitation is transporting the substrates or products across the cell membrane. Whole cell reactions are in general performed with low concentration tolerance as substrates or products may be toxic at higher concentrations. Whole cells biotransformations are associated with expensive equipment, tedious workup due to larger culture volumes, low productivity due to above mentioned factors. Byproduct formation is more probable in whole cell mediated biotransformations. Some of the limitations can be overcome by using recombinant whole cells by knocking-out irrelevant enzymes.

For highly optimized industrial processes, immobilized cells show advantage over growing cells.^{17,18}

2.2.3 Isolated enzymes vs. whole-cells

Both isolated enzymes and whole-cell mediated biocatalysis are applied in industrial as well as small scale processes. The use of whole-cells is economic and relatively easier to start with as compared to purified enzymes.¹⁹ In the course of process development, generally, the identification of best suited enzyme starts with whole cell biotransformations. After the enzyme is identified, it is overexpressed and conditions are optimized accordingly. The option to use the enzyme in isolated form or overexpressed in the whole cells is considered afterwards. Whole cells provide the best natural environment plus all necessary cofactors

¹⁷ [17] M. D. Mihovilovic and D. A. Bianchi in *Biooxidations in Chiral Synthesis*, Vol. Asymmetric Organic Synthesis with Enzymes, Wiley-VCH, **2008**, pp. 229-274.

¹⁸ [18] P. Zajkoska, M. Rebroš and M. Rosenberg, *Applied Microbiology and Biotechnology* **2013**, *97*, 1441-1455.

¹⁹ [19] T. Ishige, K. Honda and S. Shimizu, *Current Opinion in Chemical Biology* **2005**, *9*, 174-180.

required for the transformation and are sometimes more beneficial. For cleaner reactions and better activity isolated enzymes are beneficial. In some cases where the transfer of substrate to the active site is a limiting factor Crude Cell Extract (CCE) biotransformations are employed as alternative to the enzyme purifications.

2.2.4 Hybrid catalysis

Another strategy to bridge the gap between classical chemical reactions and biocatalysis is to synthesize hybrid catalytic systems by the combination of enzymatic and chemical catalysis. By studying the structure activity relation of biocatalysts, hybrid systems can be designed in order to expand the spectrum of transformations under more challenging conditions. Various biopolymers, bio-inspired ligands, and supramolecular catalysts have been designed to perform stereoselective reactions. A few research articles about using biocatalysts in their non-natural environment in order to perform chemical reactions, have been reported.^{20,21,22,23}

2.2.5 Advantages and disadvantages of biocatalysis

2.2.5.1 Advantages

Enzymes are highly chemoselective and act on the single type of functional group and have little effect on other sensitive functional groups in the substrate. This helps save many steps of protection deprotection especially in the field of total synthesis. As mentioned earlier in section 2.1.1 enzymes also express high degree of regio- and stereoselectivity due to their complex 3D structures. Enzymes are natural chiral catalysts and hence highly enantioselective while performing the transformations.

Enzymes have low waste component favorable reaction mass efficiency, E-value, and effective mass yields. Enzymatic processes are typically 10^8 – 10^{10} faster than non-catalyzed ones.²⁴ Enzymes can favor the rate enhancements by magnitude of up to $10^{17,25,26,27}$ Chemical

²⁰ [20] M. D. Mihovilovic, *J. Chem. Technol. Biotechnol.* **2007**, *82*, 1067-1071.

²¹ [21] E. Sansiaume-Dagousset, A. Urvoas, K. Chelly, W. Ghattas, J.-D. Marechal, J.-P. Mahy and R. Ricoux, *Dalton Trans.* **2014**, *43*, 8344-8354.

²² [22] A. Onoda and T. Hayashi, *Yuki Gosei Kagaku Kyokaishi* **2013**, *71*, 452-460.

²³ [23] M. Raynal, P. Ballester, A. Vidal-Ferran and P. W. N. M. van Leeuwen, *Chemical Society Reviews* **2014**, *43*, 1734-1787.

²⁴ [24] K. Faber, *Biotransformations in Organic Chemistry: A Textbook*, Springer, Newyork, **2011**, p. 3

²⁵ [25] D. L. Zechel and S. G. Withers, *Accounts of Chemical Research* **2000**, *33*, 11-18.

²⁶ [26] F. M. Menger, *Accounts of Chemical Research* **1993**, *26*, 206-212.

²⁷ [27] R. Wolfenden and M. J. Snider, *Accounts of Chemical Research* **2001**, *34*, 938-945.

catalysts in comparison are generally used in mole percentage of 0.1-1 whereas enzymes are used in mole percentage of 10^{-3} - 10^{-4} .

Enzymes work under mild reaction conditions in the range of neutral pH (5-8) and temperature 20-40°C. Biocatalytic media and enzymes used are non-toxic, environmentally acceptable and wastes produced by enzymatic reactions are biodegradable.

Enzymes are compatible with each other, hence, enabling the design of reactions in sequence without doing multistep purifications. Sometimes it is also possible to recycle and reuse the enzymes.

Enzymes can catalyze a wide range of substrates and almost every type of organic reaction can be carried out biocatalytically.

Enzymes are not restricted to their natural role and accept large substrate variety, can be over-expressed by directed evolution for increased efficiency towards substrates.

2.2.5.2 Disadvantages

Major advantage of enzymes is also their major drawback as they are available in only one enantiomeric form in nature. It is very difficult to convert the other enantiomer of substrate. In order to perform that reaction one has to search for the enzyme with opposite stereoselectivity. However, sometimes it is possible to use stereo-complementary enzymes²⁸ learning from how nature deals with mirror image substrates.

Enzymes work in aqueous environment and most organic reactions are least favored in aqueous conditions as organic compounds have poor water solubility. They have limitation to work in organic solvents with few exceptions. They work in narrow range of temperature and pH and slow reaction rates cannot be modified as enzymes denature at higher temperatures which kills their efficiency.

Enzymes act best as purified form but their limited availability and difficulty in purification makes it difficult to utilize them to their full potential.

Enzymes are prone to substrate and/or product inhibition that forces us to run the reactions at higher volumes to keep lower concentrations. There are some workarounds in order to

²⁸ [28] P. F. Mugford, U. G. Wagner, Y. Jiang, K. Faber and R. J. Kazlauskas, *Angewandte Chemie International Edition* **2008**, *47*, 8782-8793.

overcome this problem including gradual addition of substrate and removal of product by different means.

Enzymes are bound to their natural cofactors [heme, flavin, NAD(P)H] that cannot be replaced with chemical substituents. Cofactors are unstable and very expensive and recycling/regeneration is difficult. Enzymes also depend on co-substrate and have limited stability in isolated state. Side reactions such as hydrolysis, polymerization or racemization can also occur resulting in product mixtures. Cost for specialized equipment for fermentations is also high as comparative to traditional chemical reactions and high investments are need into training for new techniques.

2.3 Modern aspects of biocatalysis

Biocatalytic transformations can be performed in more challenging conditions and have emerged as very useful tool for carrying out a broad range of chemical reactions. Use of enzymes in organic solvents, ionic liquids, SCFs is emerging field of biocatalysis. Various methods have been developed including enzyme immobilizations, cross-linked enzyme aggregates, enzyme cascades,²⁹ and enzyme combinations for specialized processes.

Active research areas include genetic engineering of enzymes, overexpressing the specific enzyme in microorganisms, knocking-out the non-relevant enzyme production, increasing enzyme stability under thermal and reaction conditions, substrate concentration tolerance and specificity, broad temperature and pH range, and in non-aqueous solvents.^{30,31} Biocatalysis and biotransformations have become accepted synthetic tools for synthetic organic chemistry. Rational design and directed evolution is key research area for finding best suited counter process for the existing chemicals reactions and to establish new processes.^{32,33,34} Molecular biology tools available are overexpression and gene knockout to modify the expression level of the enzymes. The design of mutant strains lacking competing

²⁹ [29] N. Oberleitner, C. Peters, J. Muschiol, M. Kadow, S. Sass, T. Bayer, P. Schaaf, N. Iqbal, F. Rudroff, M. D. Mihovilovic and U. T. Bornscheuer, *ChemCatChem* **2013**, *5*, 3524-3528.

³⁰ [30] F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey and B. Buhler, *Green Chemistry* **2011**, *13*, 226-265.

³¹ [31] L. M. Newman, H. Garcia, T. Hudlicky and S. A. Selifonov, *Tetrahedron* **2004**, *60*, 729-734.

³² [32] M. D. Mihovilovic, F. Rudroff, A. Winninger, T. Schneider, F. Schulz and M. T. Reetz, *Organic Letters* **2006**, *8*, 1221-1224.

³³ [33] M. T. Reetz in *Directed Evolution as a Means to Engineer Enantioselective Enzymes*, Vol. Asymmetric Organic Synthesis with Enzymes, Wiley-VCH, **2008**, pp. 21-63.

³⁴ [34] S. E. Milnera and A. R. Maguire, *ARKIVOC* **2012**, 321-382.

enzymes to those involved in a certain process often results in a significant improvement in the biotransformation selectivity.

Ionic Liquids are less volatile, are chemically and thermally stable and are reported to be environmentally less toxic that makes them alternative to the classical organic solvents. There are various biocatalytic reactions investigated in ionic liquids and in most cases show improved selectivity, enzyme stability, and activity. These reactions include lipases, proteases, oxidoreductases,^{35,36} alcohol dehydrogenases, peroxidases, hydroxynitrile lyases, and cytochrome p450.³⁷ Furthermore, attempts have been made to design cofactor independent reactions,^{38,39,40} to combine bio- and chemo-catalysis⁴¹ and to synthesize bio-inspired catalysis as well.^{42,43}

2.4 Green chemistry and biocatalysis

Green chemistry principles play key role in meeting the environmental and economic goals simultaneously. The systematically set twelve principles are key guidelines for a chemical process to meet the challenge of green chemistry.⁴⁴ One technology that has become a central part of green chemistry is biocatalysis. Biocatalysis are in alignment with green chemistry guidelines in several ways as these reactions fulfill the requirements for green chemistry to a greater extent.^{45,46} Biocatalysis comes is closest agreement with the green chemistry guidelines and the key principles of green chemistry as they are met by biocatalysis are summarized in the table 2-1.^{47,48,49}

³⁵ [35] C. C. R. Allen, C. J. Boudet, C. Hardacre and M. E. Migaud, *RSC Advances* **2014**, *4*, 19916-19924.

³⁶ [36] C. Hardacre, M. E. Migaud and K. A. Ness, *New Journal of Chemistry* **2012**, *36*, 2316-2321.

³⁷ [37] K. L. Tee, D. Roccatano, S. Stolte, J. Arning, B. Jastorff and U. Schwaneberg, *Green Chemistry* **2008**, *10*, 117-123.

³⁸ [38] S. Fetzner and R. A. Steiner, *Appl. Microbiol. Biotechnol.* **2010**, *86*, 791-804.

³⁹ [39] S. Fetzner, *Applied Microbiology and Biotechnology* **2002**, *60*, 243-257.

⁴⁰ [40] I. Bauer, N. Max, S. Fetzner and F. Lingens, *European Journal of Biochemistry* **1996**, *240*, 576-583.

⁴¹ [41] A. C. Marr and S. Liu, *Trends Biotechnol.* **2011**, *29*, 199-204.

⁴² [42] P. G. Andersson and Editor, *Innovative Catalysis in Organic Synthesis: Oxidation, Hydrogenation, and C-X Bond Forming Reactions*, Wiley-VCH, **2012**, p. 356 pp.

⁴³ [43] I. Garcia-Bosch, I. Prat, X. Ribas and M. Costas in *Bioinspired oxidations catalyzed by nonheme iron and manganese complexes*, Vol. Innovative Catalysis in Organic Synthesis, Wiley-VCH, **2012**, pp. 27-46.

⁴⁴ [44] P. Anastas and N. Eghbali, *Chemical Society Reviews* **2010**, *39*, 301-312.

⁴⁵ [45] T. Hudlicky, D. A. Frey, L. Koroniak, C. D. Claeboe and L. E. Brammer Jr, *Green Chemistry* **1999**, *1*, 57-59.

⁴⁶ [46] N. Ran, L. Zhao, Z. Chen and J. Tao, *Green Chemistry* **2008**, *10*, 361-372.

⁴⁷ [47] P. W. Sutton, J. P. Adams, I. Archer, D. Auriol, M. Avi, C. Branneby, A. J. Collis, B. Dumas, T. Eckrich, I. Fotheringham, R. ter Halle, S. Hanlon, M. Hansen, K. E. Holt-Tiffin, R. M. Howard, G. W. Huisman, H. Iding, K. Kiewel, M. Kittelmann, E. Kupfer, K. Laumen, F. Lefèvre, S. Luetz, D. P. Mangan, V. A. Martin, H.-P. Meyer, T. S. Moody, A. Osorio-Lozada, K. Robins, R. Snajdrova, M. D. Truppo, A. Wells, B. Wirz and J. W. Wong in *Biocatalysis in the Fine Chemical and Pharmaceutical*

Table 2-1 Biocatalysis alignment with green chemistry

Entry	Green chemistry principle	Biocatalysis
1	Prevent waste	Can enable new, more sustainable routes to APIs effectively reducing level of waste
2	Atom economy	Highly efficient and have higher atom economy
3	Less hazardous (less toxic reagents and intermediates) chemical syntheses	Generally low toxicity
4	Designing safer (less toxic) chemicals	Less to no impact
5	Safer solvents and auxiliaries	Often performed in water; when solvents are used they are generally Class I or II.
6	Design for energy efficiency	Usually performed slightly above room temperature.
7	Use of renewable materials	Biocatalysts are renewable
8	Reduce derivatization steps	Chemo-, regio- & enantio-selective nature of enzymes often obviates need for protecting groups
9	Catalytic (preferred over stoichiometric reagents)	Catalytic
10	Design for degradation	Biocatalysts are degradable in the environment
11	Real-time analysis for pollution (and hazard) prevention	No impact on environment
12	Inherently safer chemistry for accident prevention	Generally performed under mild conditions where risk of hazard is minimal

Table adapted and reproduced by Sutton, Tang, redox biocatalysis, and Anastas.⁴⁹

Industries, Vol. Practical Methods for Biocatalysis and Biotransformations 2, John Wiley & Sons, **2012**, pp. 1-59.

⁴⁸ [48] S. Y. Tang, R. A. Bourne, R. L. Smith and M. Poliakoff, *Green Chemistry* **2008**, *10*, 268-269.

⁴⁹ [49] P. T. Anastas and J. B. Zimmerman, *Environmental Science & Technology* **2003**, *37*, 94A-101A.

2.5 Redox biocatalysis

Major success factors for the application of biooxidation methods have always been their efficiency, safety, selectivity, and step economy. Biooxidations become more vital for the reactions when either very few alternatives in classic chemical oxidations are available, or when no chemical transformations have been discovered.

The use of safe, non-toxic and biologically benign bio-oxidants in aqueous solvent systems renders them to be the method of choice in many cases. Biooxidations and the availability of microbial biocatalysts have enlarged the scope of microbial biotransformations to a reasonable extent.⁵⁰ Molecular oxygen being a cheap and environmentally benign reagent cannot directly be used efficiently in oxidation reactions of non-activated organic compounds⁵¹ whereas microbial oxidations do the job quite effectively.

In the development of highly efficient synthetic pathways, biooxidations allow for shorter reaction sequences towards complex target molecules by partially avoiding the protection and deprotection steps. The adaptability and high regio-⁵² and stereoselectivity⁵³ of enzymes towards non-natural substrates enables us to exploit them in a highly efficient and predictable manner.

Due to these benefits the applications of large variety of diverse biooxidation catalysts has been actively studied as evident in the literature published in recent years.^{13,54,55} Redox biocatalysis is performed by a class of enzymes called oxidoreductases and these are sub-classified into following four categories;⁵⁶

- Oxygenases
- Oxidases
- Peroxidases
- Dehydrogenases

⁵⁰ [50] R. Wohlgemuth in *Oxidation by Microbial Methods, Vol.* (Ed. P. Knochel), Comprehensive Organic Synthesis II (Second Edition), Elsevier, Amsterdam, **2014**, pp. 121-144.

⁵¹ [51] J.-E. Baeckvall and Editor, *Modern Oxidation Methods*, Wiley-VCH, **2010**, p. 465 pp.

⁵² [52] D. J. Ferraro, A. L. Okerlund, J. C. Mowers and S. Ramaswamy, *J. Bacteriol.* **2006**, *188*, 6986-6994.

⁵³ [53] O. Uhlik, M.-C. Leewis, M. Strejcek, L. Musilova, M. Mackova, M. B. Leigh and T. Macek, *Biotechnology Advances* **2013**, *31*, 154-165.

⁵⁴ [54] A. S. Bommarius and B. R. Riebel in *Biocatalysis, Vol.* Biocatalysis, Wiley-VCH, **2004**, p. 611.

⁵⁵ [55] D. J. Leak, Y. Yin, J.-J. Zhang and N.-Y. Zhou in *Aromatic Oxidations, Vol.* Eds.: K. Drauz, H. Groeger and O. May), Enzyme Catalysis in Organic Synthesis, Wiley-VCH, **2012**, pp. 1487-1533.

⁵⁶ [56] D. Gamemara, G. A. Seoane, P. Saenz-Méndez and P. D. de María in *Enzymes Involved in Redox Reactions: Natural Sources and Mechanistic Overview, Vol.* Redox Biocatalysis, John Wiley & Sons, Inc., **2012**, pp. 1-85.

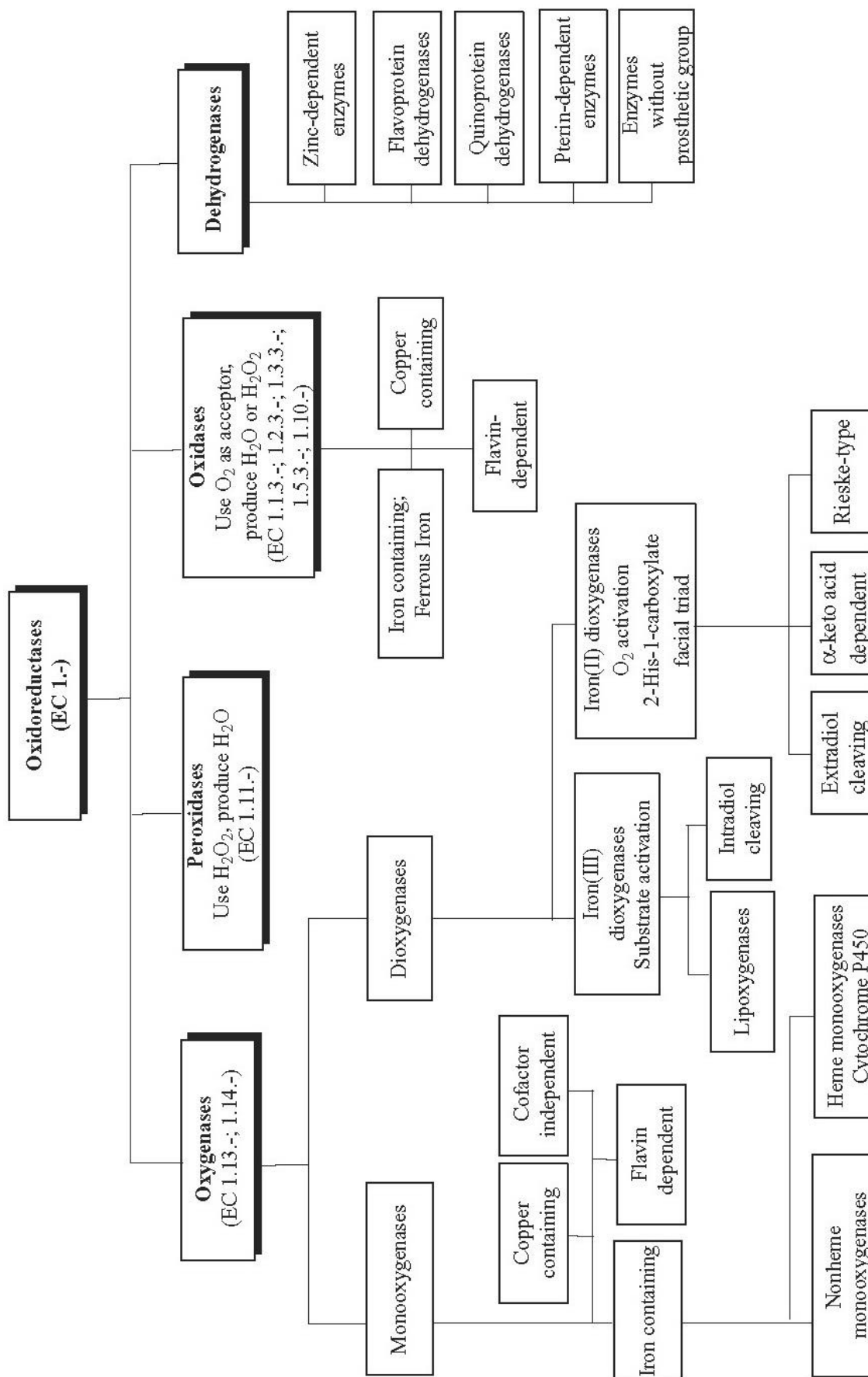
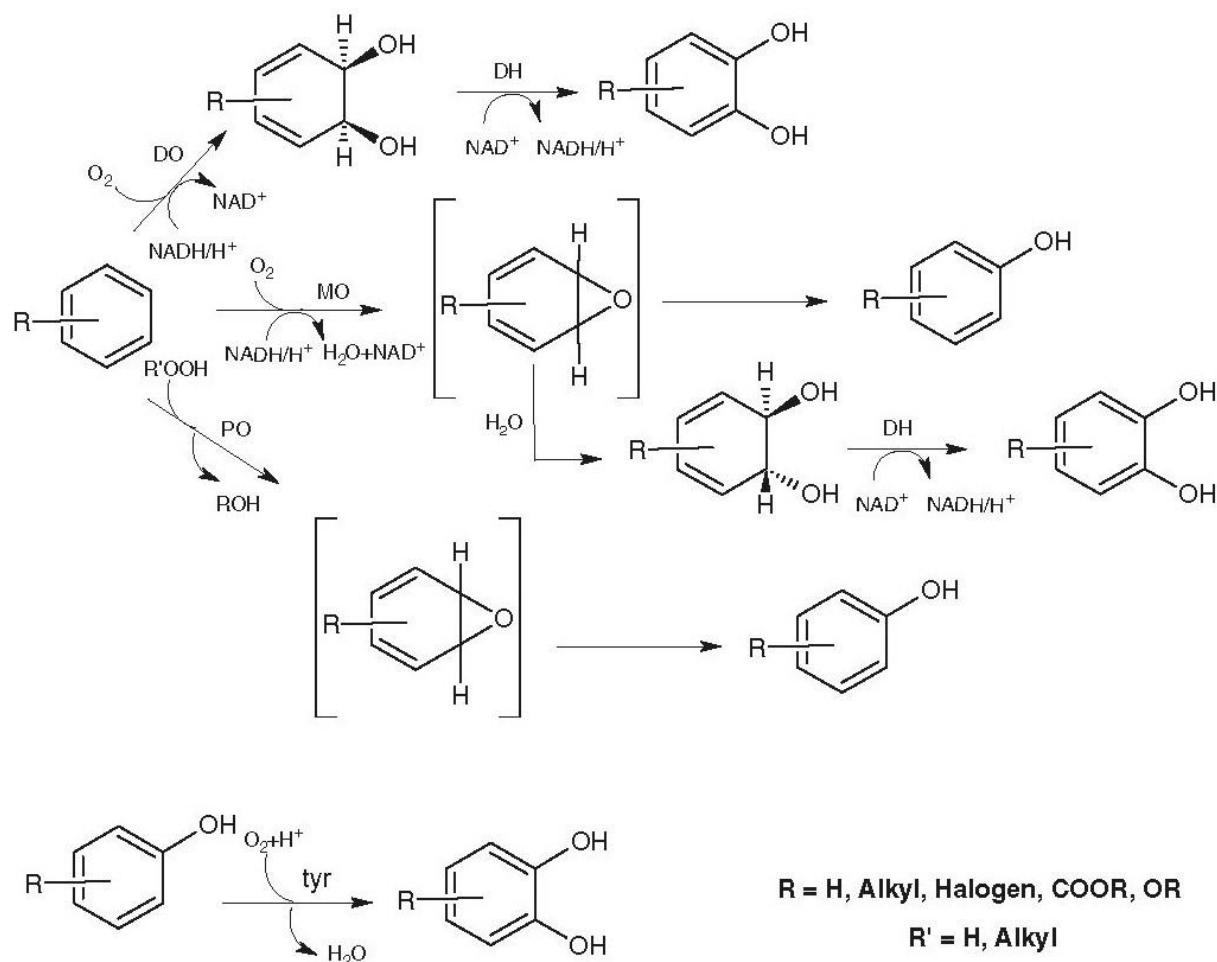


Fig. 2-2 Classification of redox biocatalysis adapted from Redox Biocatalysis⁵⁶

A recent review by Gennaro *et al.*⁵⁷ briefly summarized the role of oxidoreductases in hydroxylation and detoxification of PAHs. Scheme 2-1 gives an overview of principal transformations of aromatic compounds by dioxygenases, monooxygenases, peroxidases, tyrosinases, and dehydrogenases to form mono or dihydroxy arenes.



Scheme 2-1 Hydroxylation of AHs by oxidoreductases adapted from Gennaro⁵⁷

2.6 Monooxygenases

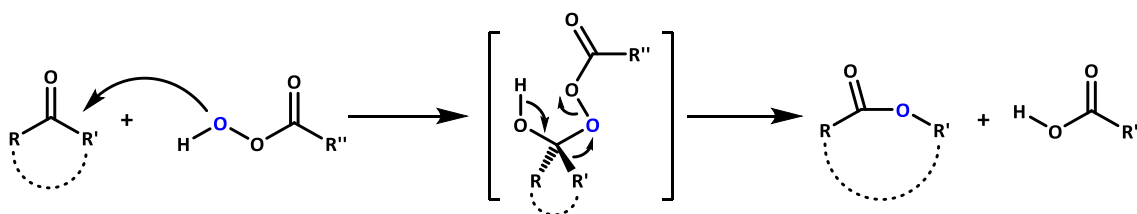
Monooxygenases is the group of enzymes that incorporate one oxygen atom from molecular oxygen into the substrate; the other is reduced at the expense of a donor (usually NADH or NADPH) to form water. Monooxygenases contain either flavin or iron in their active sites. Iron containing monooxygenases are divided into non-heme monooxygenases mainly found in prokaryotes and heme monooxygenases as cytochrome P450s. Monooxygenases perform following transformations on the substrate;

⁵⁷ [57] P. Di Gennaro, A. Bargna and G. Sello, *Applied Microbiology and Biotechnology* **2011**, *90*, 1817-1827.

- Monohydroxylations
- Epoxidations
- Baeyer-Villiger Oxidations (BVOs)

2.6.1 Baeyer-Villiger monooxygenases (BVMOs)

Baeyer-Villiger oxidation is the oxidative transformation of ketone into either lactone or ester by the treatment with peroxy acid or hydrogen peroxide.⁵⁸ Classic chemical reactions use reagents such as meta-chloroperbenzoic acid, peroxyacetic acid, or other modified peroxyacids.⁵⁹ Baeyer-Villiger oxidation is very important in synthetic chemistry due to its highly predictable nature. To perform this reaction in more enantioselective and greener fashion, biocatalytic transformation can be employed.⁶⁰



Scheme 2-2 General mechanism of peroxyacid oxidation

Monooxygenases that incorporate single oxygen into ketone substrate to perform Baeyer-Villiger oxidation are termed as Baeyer-Villiger monooxygenases (BVMOs). BVMOs (EC 1.14.13.x) are abundantly found in prokaryotes and fungi playing major role in their catabolic pathways and enabling them to grow on diverse alternative carbon sources. BVMOs are found in abundance in waste water facilities of chemical industries and special techniques are needed to isolate the expression strains. First BVMOs were isolated and characterized in 1960s.^{61,62} A recent book gives comprehensive overview about scope and applications of BOMVs.⁶³

BVMOs can be classified in two groups: Type I BVMOs are flavin dependent enzymes and contain flavin adenine dinucleotide (FAD) as cofactor, consist of identical

⁵⁸ [58] A. von Baeyer and V. Villiger, *Chem. Ber.* **1899**, 32, 3625-3633.

⁵⁹ [59] H. Hussain, A. Al-Harrasi, I. R. Green, I. Ahmed, G. Abbas and N. U. Rehman, *RSC Advances* **2014**, 4, 12882-12917.

⁶⁰ [60] N. M. Kamerbeek, D. B. Janssen, W. J. H. van Berkel and M. W. Fraaije, *Advanced Synthesis & Catalysis* **2003**, 345, 667-678.

⁶¹ [61] H. E. Conrad, R. DuBus, M. J. Namtvedt and I. C. Gunsalus, *Journal of Biological Chemistry* **1965**, 240, 495-503.

⁶² [62] P. W. Trudgill, R. DuBus and I. C. Gunsalus, *Journal of Biological Chemistry* **1966**, 241, 4288-4290.

⁶³ [63] M. D. Mihovilovic in *Baeyer-Villiger Oxidations*, Vol. 3rd Eds.: K. Drauz, H. Groeger and O. May), Enzyme Catalysis in Organic Synthesis, Wiley-VCH, **2012**, pp. 1439-1485.

subunits and NAD(P)H is required in order to activate flavin prosthetic group for the reaction with molecular oxygen. Type II BVMOs contain flavin mononucleotide (FMN) as cofactor, use NADH as electron donor and are composed of $\alpha\beta$ trimmers.^{64,65}

2.6.1.1 Synthetic applications of BVMOs

BVMOs have been successfully applied to number of synthetic processes including desymmetrizations, kinetic resolutions, and regiodivergent reactions,^{66,67,68} esters and lactone syntheses, and synthesis of aroma compounds.^{69,70}

2.6.2 Monooxygenases with epoxide hydrolases

In order to produce enantiomerically pure *trans*-diols enzyme combination of monooxygenase and epoxide hydrolase can be applied. Monooxygenases can be used to perform stereospecific direct epoxidation of alkenes.⁷¹ Epoxide hydrolases (EH) perform hydrolysis of an epoxide via S_N2 type ring opening leading to the formation of corresponding *trans*-diols. Degradation of PAHs can proceed via dioxygenase catalyzed formation of *cis*-diols resulting from a dioxetane intermediate or can proceed via monooxygenase catalyzed epoxidation followed by epoxide-hydrolase catalyzed ring-opening to the *trans*-diols.^{72,73} In contrast to oxygenases, hydrolases don't need cofactors, are easy to use, can also work in organic solvents, and are commercially available.

A recent literature gives an overview about synthesis of *trans*-diols by combining monooxygenase (MO) and epoxide hydrolases (EH) as one pot synthesis.^{74,75,76,77}

⁶⁴ [64] A. Willetts, *Trends Biotechnol.* **1997**, *15*, 55-62.

⁶⁵ [65] A. Riebel, M. J. Fink, M. D. Mihovilovic and M. W. Fraaije, *ChemCatChem* **2014**, *6*, 1112-1117.

⁶⁶ [66] G. de Gonzalo, M. D. Mihovilovic and M. W. Fraaije, *ChemBioChem* **2010**, *11*, 2208-2231.

⁶⁷ [67] J. Rehdorf and U. T. Bornscheuer, *Encyclopedia of Industrial Biotechnology* **2010**, pp. 3572-3607.

⁶⁸ [68] K. Balke, M. Kadow, H. Mallin, S. Sass and U. T. Bornscheuer, *Org. Biomol. Chem.* **2012**, *10*, 6249-6265.

⁶⁹ [69] M. J. Fink, F. Rudroff and M. D. Mihovilovic, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6135-6138.

⁷⁰ [70] H. M. Dudek, M. J. Fink, A. V. Shivange, A. Dennig, M. D. Mihovilovic, U. Schwaneberg and M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 4009-4020.

⁷¹ [71] D. Chang, J. Zhang, B. Witholt and Z. Li, *Biocatal. Biotransform.* **2004**, *22*, 113-131.

⁷² [72] U. T. Bornscheuer and R. J. Kazlauskas in *Epoxide Hydrolases*, Vol. Hydrolases in Organic Synthesis, Wiley-VCH, **2006**, pp. 215-226.

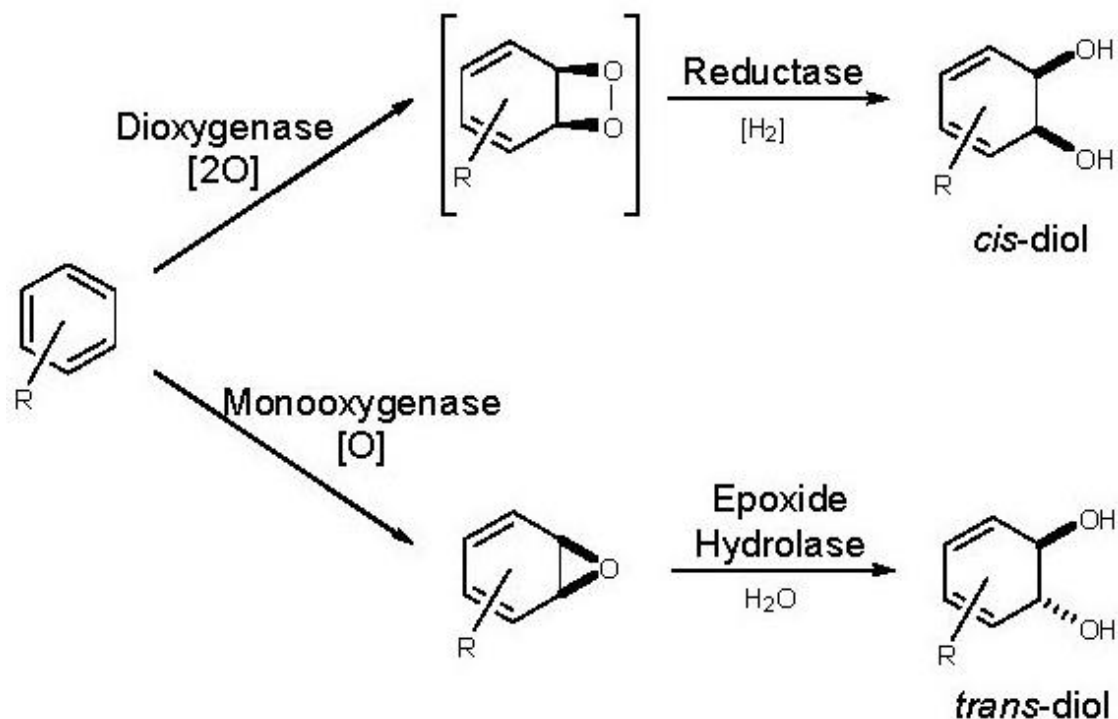
⁷³ [73] H. Zheng, D. Kahakeaw, J. P. Acevedo and M. T. Reetz, *ChemCatChem* **2010**, *2*, 958-961.

⁷⁴ [74] G. Bellucci, G. Berti, G. Catelani and E. Mastroilli, *J. Org. Chem.* **1981**, *46*, 5148-5150.

⁷⁵ [75] Y. Xu, X. Jia, S. Panke and Z. Li, *Chem. Commun.* **2009**, 1481-1483.

⁷⁶ [76] Y. Xu, A. Li, X. Jia and Z. Li, *Green Chem.* **2011**, *13*, 2452-2458.

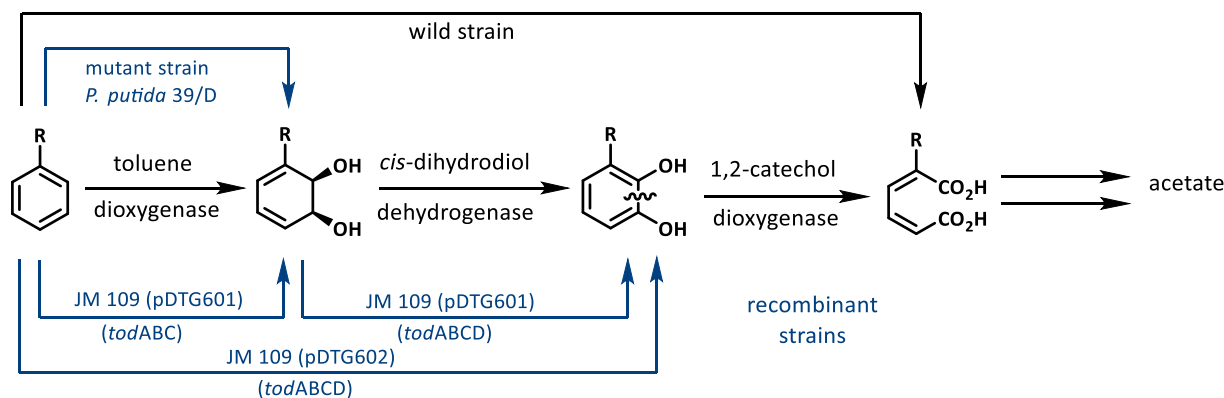
⁷⁷ [77] S. Wu, Y. Chen, Y. Xu, A. Li, Q. Xu, A. Glieder and Z. Li, *ACS Catal.* **2014**, *4*, 409-420.



Scheme 2-3 *cis*- and *trans*- diols resulting from aerobic degradation of PAHs⁷²

2.7 Dioxygenases

Ring hydroxylating oxygenases (RHOs) are capable to perform dearomatization reactions on aryl and hetaryl ring systems with concomitant stereoselective introduction of two alcohol functionalities. Dioxygenases catalyze first key step of *cis*-dihydroxylation of PAHs and NPAHs generating two chiral centers in the process of degradation by microorganisms. Within wild-type organisms this process is usually complemented by action of a dihydrodiol-dehydrogenase (DD), ultimately generating a rearomatized product and loss of chirality. These hydroxylated aromatic products then undergo ortho cleavage to the corresponding aliphatic acids. Hydration and retro-aldol reactions then provide acetate as an energy and carbon source (scheme 2-4). The overall biotransformation sequence leads to an increase of hydrophilicity of aromatic xenobiotics to be excreted more readily upon detoxification.



Scheme 2-4 Metabolism of aromatic compounds by soil organisms adapted from Hudlicky¹⁰¹

Microbial dihydroxylation of toluene by toluene dioxxygenase (TDO) to form catechol was first time reported in 1968 by Gibson.⁷⁸ This enzyme combination of dioxxygenase and dihydrodiol-dehydrogenase was also exploited synthetically to obtain several catechols with the loss of chirality.⁷⁹ In context of asymmetric synthesis, it is mandatory to stop the conversion at the dihydro-diol stage, which was realized in the early days by using mutant strains deficient in enzymes responsible for the rearomatization. This could be achieved by genetically modifying the wild type microorganisms both by suppressing or completely removing the diol-dehydrogenase activity and by overexpressing the dioxxygenases component. This approach allows cis-dihydrodiol metabolites to be intercepted without catechol formation. In 1970 Gibson used recombinant *P. putida* 39/D in order to remove DD and use dioxxygenase in order to stop the process at oxygenation stage.⁸⁰ These mutant type dioxxygenase producing strains were successfully applied to synthesize a large number of chiral synthons.⁸¹ Process of dihydroxylation in the whole-cell mutant strains regardless of NDO, TDO, BPDO, BZDO, and CDO was published by Parales⁸² and Takahashi for BPDO.⁸³ Dioxxygenases have been engineered to display modified selectivity and improved efficiency, particularly using site-directed mutation and direct-evolutions methods. Alternatively,

⁷⁸ [78] D. T. Gibson, J. R. Koch and R. E. Kallio, *Biochemistry* **1968**, 7, 2653-2662.

⁷⁹ [79] V. P. Bui, T. V. Hansen, Y. Stenstrom and T. Hudlicky, *Green Chemistry* **2000**, 2, 263-265.

⁸⁰ [80] D. T. Gibson, M. Hensley, H. Yoshioka and T. J. Mabry, *Biochemistry* **1970**, 9, 1626-1630.

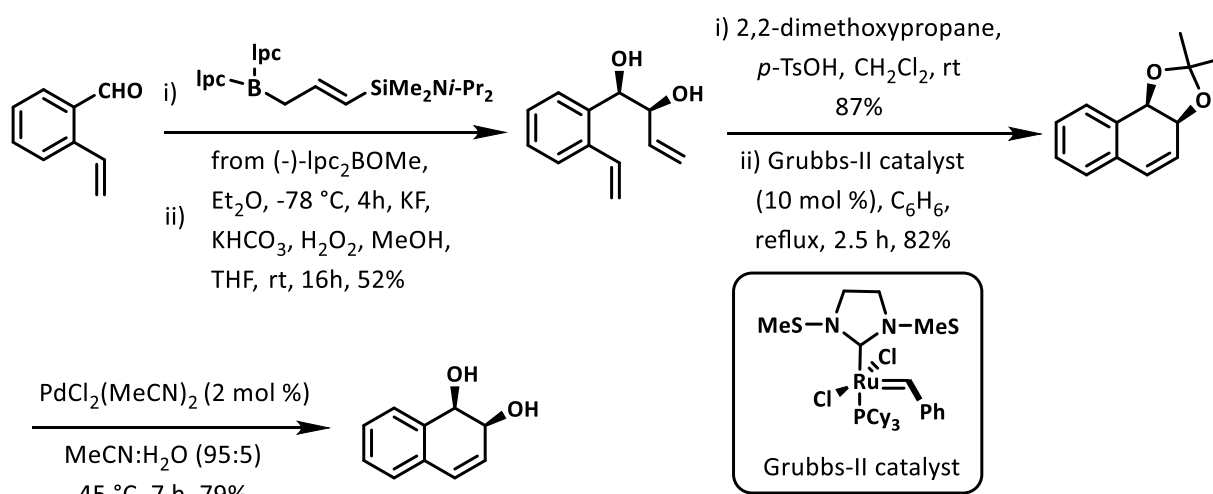
⁸¹ [81] D. R. Boyd and G. N. Sheldrake, *Nat. Prod. Rep.* **1998**, 15, 309-324.

⁸² [82] R. E. Parales, S. M. Resnick, C.-L. Yu, D. R. Boyd, N. D. Sharma and D. T. Gibson, *J. Bacteriol.* **2000**, 182, 5495-5504.

⁸³ [83] N. Misawa, K. Shindo, H. Takahashi, H. Suenaga, K. Iguchi, H. Okazaki, S. Harayama and K. Furukawa, *Tetrahedron* **2002**, 58, 9605-9612.

competing proteins can be suppressed by gene knockout methods. Sometimes a combination of both strategies can be helpful in particular cases.^{84,85}

Chemical equivalent of dihydroxylation with such an efficient enantioselectivity is not trivial task, at all. Chemical oxidation to synthesize enantioselective diols represents a very difficult transformation.⁸⁶ Chemical synthesis of enantiomerically pure cis-diols can be compared by chemical and biocatalytic methods by following example of naphthalene diol synthesis.⁸⁷



Scheme 2-5 Chemical equivalent of dihydroxylation adapted from Mukherjee⁸⁷

2.8 Classification of dioxygenases

Classification of RHOs is useful for understanding the relationship between various aspects of sequence, structure, function and evolution. Dioxygenases are named after the substrate used as carbon source but are not limited to converting only that substrate and over 100 dioxygenases have been discovered, so far. The most widely used RHO types in biotransformations include benzene-(BDO), toluene- (TDO), biphenyl- (BPDO), chlorobenzene- (CDO), benzoic acid-(BZDO), nitrobenzene- (NBDO), and naphthalene-dioxygenase (NDO). Initial classification of dioxygenases was based on electron transfer component present in the oxygenase system. These dioxygenases were subdivided into

⁸⁴ [84] G. Carrea and S. Riva in *Medium Engineering, Vol.* Eds.: V. Gotor, I. Alfonso and E. Garcia-Urdiales), *Asymmetric Organic Synthesis with Enzymes*, Wiley-VCH, **2008**, pp. 1-20.

⁸⁵ [85] V. Gotor, I. Alfonso, E. Garcia-Urdiales and Editors, *Asymmetric Organic Synthesis with Enzymes*, Wiley-VCH, **2008**, p. 325 pp.

⁸⁶ [86] C. J. R. Bataille and T. J. Donohoe, *Chemical Society Reviews* **2011**, *40*, 114-128.

⁸⁷ [87] P. Mukherjee, S. J. S. Roy and T. K. Sarkar, *Organic Letters* **2010**, *12*, 2472-2475.

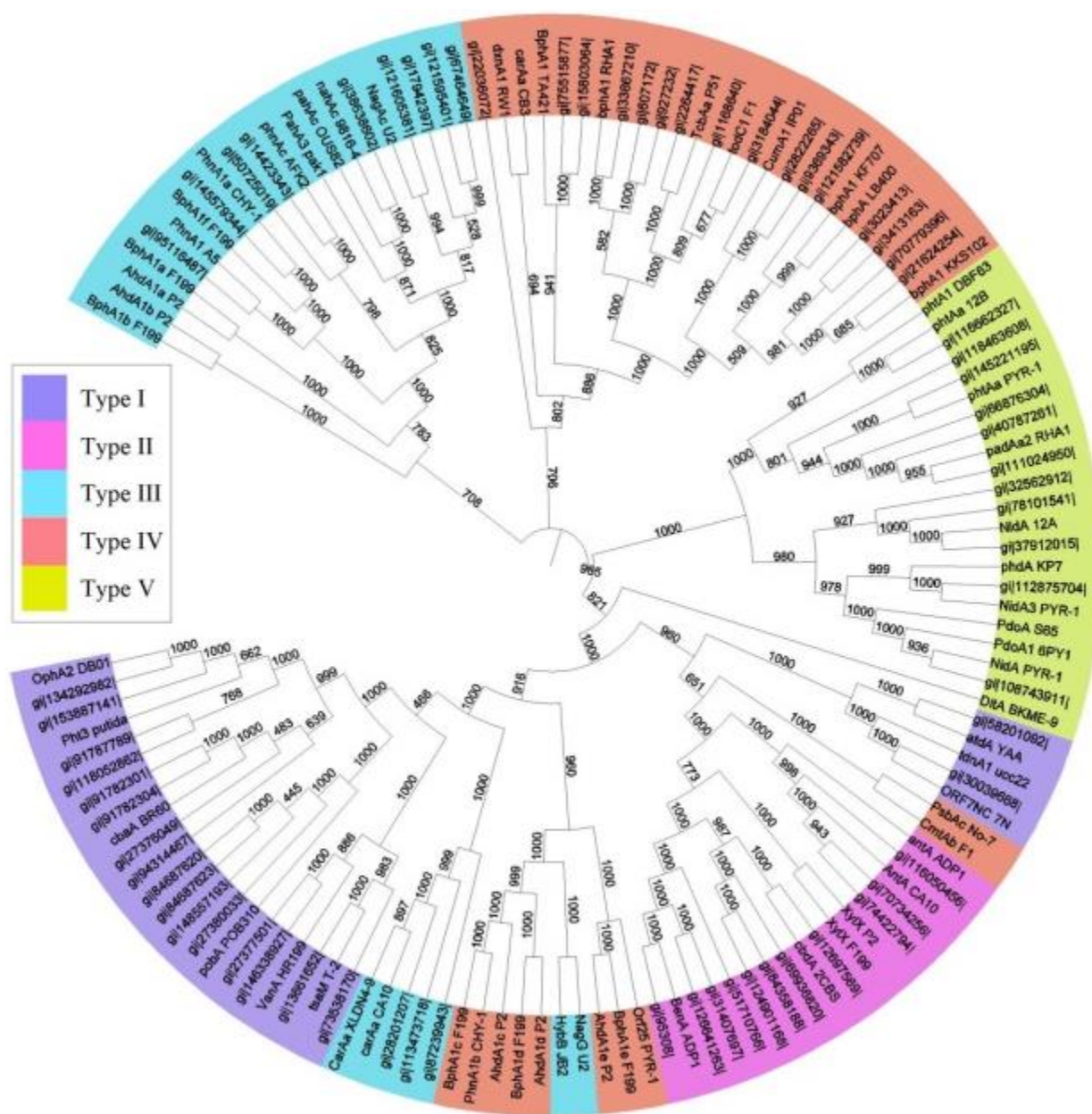


Fig. 2-4 Phylogenetic tree adapted from Kweon⁸⁸

With the development and discovery of more dioxygenase types, the phylogenetic relationship among members of this enzyme family has been expanded and RHOs are grouped into four families based on substrate specificities and many different factors.⁸⁸ This classification emphasized more on the structure-function relationship of the oxygenase

⁸⁸ [88] O. Kweon, S.-J. Kim, S. Baek, J.-C. Chae, M. D. Adjei, D.-H. Baek, Y.-C. Kim and C. E. Cerniglia, *BMC Biochem.* **2008**, *9*, 1-20.

component. Later on software was also developed in order to access the database effectively and in order to keep it in order.⁸⁹

Classification	Structure	Enzyme system
Type I	Type I $\alpha\beta$	Aniline dioxygenase (<i>Acinetobacter</i> sp. YAA) Aniline oxygenase (<i>P. putida</i> UCC22)
	Type I α	Phenoxybenzoate dioxygenase (<i>Alcaligenes</i> sp. BR60) Phenoxybenzoate dioxygenase (<i>P. pseudoalcaligenes</i> POB310) Phthalate dioxygenase (<i>P. cepacia</i> DB01) Toluenesulfonate monooxygenase (<i>C. testosterone</i> T-2)
Type II		2-Halobenzoate 1,2-dioxygenase (<i>P. cepacia</i> 2CBS) Anthranilate dioxygenase (<i>Acinetobacter</i> sp. ADP1) Benzoate 1,2-dioxygenase (<i>Acinetobacter</i> sp. ADP1)
Type III	Type III $\alpha\beta$	3,4-Dihydroxyphenanthrene dioxygenase (<i>A. faecalis</i> AFK2) Naphthalene dioxygenase (<i>Pseudomonas</i> sp. 9816-4) PAH dioxygenase (<i>P. putida</i> OUS82)
	Type III α	Carbazole 1,9a-dioxygenase (<i>P. resinovorans</i> CA10)
Type IV		Carbazole dioxygenase (<i>Sphingomonas</i> sp. CB3) Dioxin dioxygenase (<i>Sphingomonas</i> sp. RW1) Biphenyl dioxygenase (<i>Rhodococcus</i> sp. RHA1) Toluene dioxygenase (<i>P. putida</i> F1) Biphenyl dioxygenase (<i>Pseudomonas</i> sp. KKS102) Biphenyl 2,3-dioxygenase (<i>B. xenovorans</i> LB400) Biphenyl dioxygenase (<i>P. pseudoalcaligenes</i> KF707)
Type V		Phenanthrene dioxygenase (<i>Nocardioides</i> sp. KP7) Phthalate dioxygenase (<i>Terrabacter</i> sp. DBF63) Phthalate dioxygenase (<i>A. Keyseri</i> 12B) Phthalate dioxygenase (<i>M. vanbaalenii</i> PYR-1) Phthalate dioxygenase (<i>Rhodococcus</i> sp. RHA1)

Fig. 2-5 Types of RHOs adapted from Kweon⁸⁸

The classification of dioxygenases as given by Parales⁹⁰ is as follows,

Group I: phthalate dioxygenases

These are two-component enzymes, each consisting of an *an* oxygenase component (lacking β subunits) and a reductase component. Details about α and β subunits is given in group III naphthalene dioxygenase enzymes. The ferredoxin reductases are both composed of three distinct domains, for binding a plant-type [2Fe–2S] center, NAD, and flavin (FMN in PDR; FAD in BenC)

⁸⁹ [89] S. Baek, O. Kweon, S.-J. Kim, D.-H. Baek, J. J. Chen and C. E. Cerniglia, *J. Microbiol. Methods* **2009**, 76, 307-309.

⁹⁰ [90] R. Parales and S. Resnick in *Aromatic Ring Hydroxylating Dioxygenases, Vol. Volume 4: Molecular Biology of Emerging Issues* Eds.: J.-L. Ramos and R. Levesque), *Pseudomonas*, Springer US, **2006**, pp. 287-340.

Group II: benzoate dioxygenases

These are also two-component enzymes consisting of dioxygenase and ferredoxin reductase part. The systematic name of this enzyme class is benzoate NADH: oxygen oxidoreductases (1,2-hydroxylating). These are also commonly used as benzoate 1,2 dioxygenases. As clear from the name itself the enzyme uses NADH, H^+ , and O_2 and benzoate substrate and converts it into 1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate and NAD^+ .

Group III: naphthalene dioxygenases

These are Rieske type RHOs and comprise of three components: (i) a reductase domain which obtains electrons from NAD(P)H; (ii) a Rieske ferredoxin component that shuttles the electrons; (iii) and an oxygenase component that performs catalysis. The reductase component of 35 kDa and contains one molecule of FAD and a plant type iron-sulfur center. The ferredoxin is a Rieske $[2Fe-2S]$ center containing monomer of 11.4 kDa. The catalytic oxygenase component is an $\alpha_3\beta_3$ hexamer consisting of large alpha and small beta subunits. Each α subunit contains two redox centers, a Rieske $[2Fe-2S]$ center and mononuclear Fe^{2+} at the active site. Both subunits are essential for the activity.

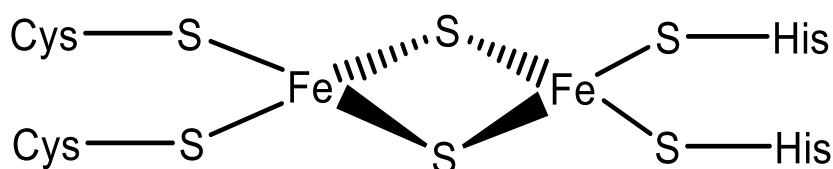


Fig. 2-6 Rieske-type $[2Fe-2S]$ cluster in the active site of NDO

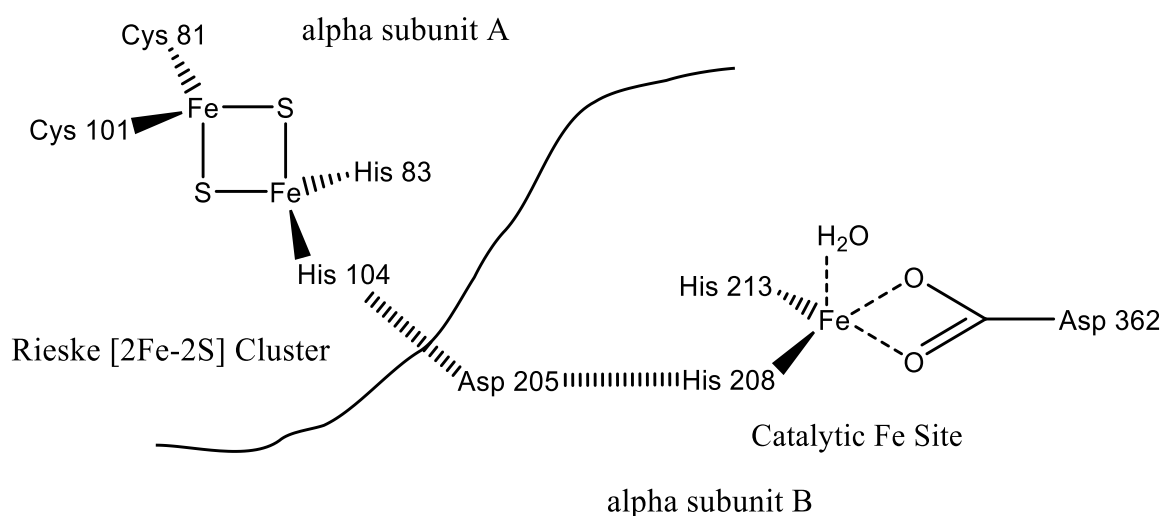


Fig. 2-7 Junction between two alpha subunits-adapted from Parales⁹⁰

The transfer of electrons takes place from the Rieske center to the mononuclear iron of the neighboring subunit via a conserved aspartate residue, which is shown to be involved in electron transport chain. Electrons are transferred sequentially from NAD(P)H to the reductase, to the ferredoxin, to the Rieske center of the oxygenase, and finally to the iron at the active site of the oxygenase. The reduced oxygenase catalyzes the addition of both atoms of O₂ to the aromatic ring. Two electrons are necessary to complete the reaction cycle.^{91,92,93} The electron transport chain showing the action of dioxygenase over the course of reaction and stoichiometry of reaction is shown in figures 2.8 and 2.9 respectively.

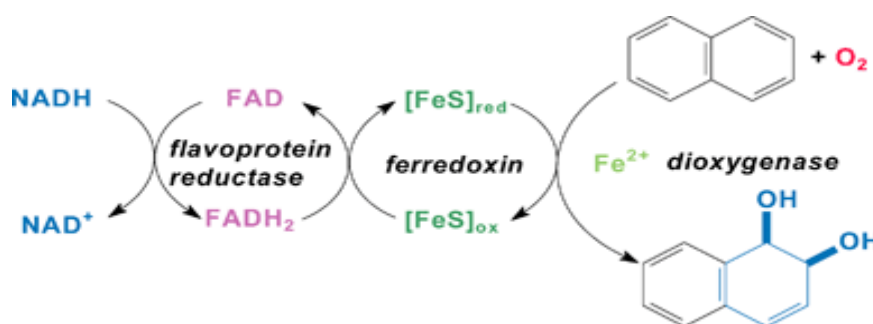


Fig. 2-8 Electron transport chain in NDO from Boyd¹⁰⁵

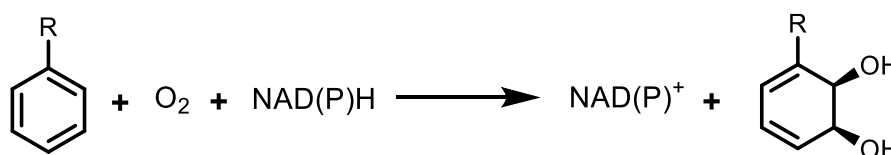


Fig. 2-9 Stoichiometry of the arene dihydroxylation

Group IV: toluene/biphenyl dioxygenases

Toluene (TDO) and biphenyl (BPDO) are 2,3-dioxygenases and are capable of accepting larger ring sizes and fused ring PAHs and NPAHs but are not limited to larger sized rings only. They can also accept smaller substrates. These enzymes are involved in toluene, xylene and biphenyl degradation, respectively.

Group V: gram positive PAH/phthalate dioxygenases

⁹¹ [91] M. Morikawa, *Appl. Microbiol. Biotechnol.* **2010**, 87, 1595-1603.

⁹² [92] S. P. de Visser, D. Kumar and Editors, *Iron-Containing Enzymes: Versatile Catalysts of Hydroxylation Reactions in Nature*, RSC, **2011**, p. 448 pp.

⁹³ [93] D. J. Ferraro, L. Gakhar and S. Ramaswamy, *Biochemical and Biophysical Research Communications* **2005**, 338, 175-190.

Some Gram-positive bacteria such as *Rhodococcus opacus*, are also capable to use PAHs (dibenzofurans, dibenzodioxins) as carbon sources, by producing a unique arene dioxygenase which catalyzes lateral dioxygenation reactions.⁹⁴ These dioxygenases are multiple component systems and were classified into a new group along with phenanthrene dioxygenases. Ferredoxin component in the dioxygenases is of [3Fe-4S] type. These are commonly found in *Nocardioides*, *Rhodococcus*, *Terrabacter*, *Arthrobacter* and other *Mycobacterium* spp.⁸⁸

Group VI: salicylate dioxygenase

A new family has emerged recently, consisting of enzymes that catalyze the oxidation at either the 1- or 5-position of salicylate as well as other substrates, and was designated as the salicylate family.⁹⁵

However, there are still members that do not belong to any of these families, such as enzymes that oxygenate aniline, dibenzodioxins, 3-phenylpropionate, or o-benzoate.⁹⁵

Because of the multicomponent nature of these ROs and the need of cofactors, the biotransformations are preferably performed using recombinant whole-cell biocatalytic systems.

2.9 Mechanism of dihydroxylation

The most difficult step in the aerobic degradation of PAHs and NPAHs is the hydroxylation of aromatic rings. Oxygenases that use non-heme Fe (II) are called Rieske-type non-heme iron aromatic ring hydroxylating oxygenases (RHO) and catalyze the stereoselective introduction of two hydroxyl groups into the aromatic ring.⁹⁶ RHOs play key role in natural degradation of PAHs and are very important in biosynthesis of secondary metabolites.

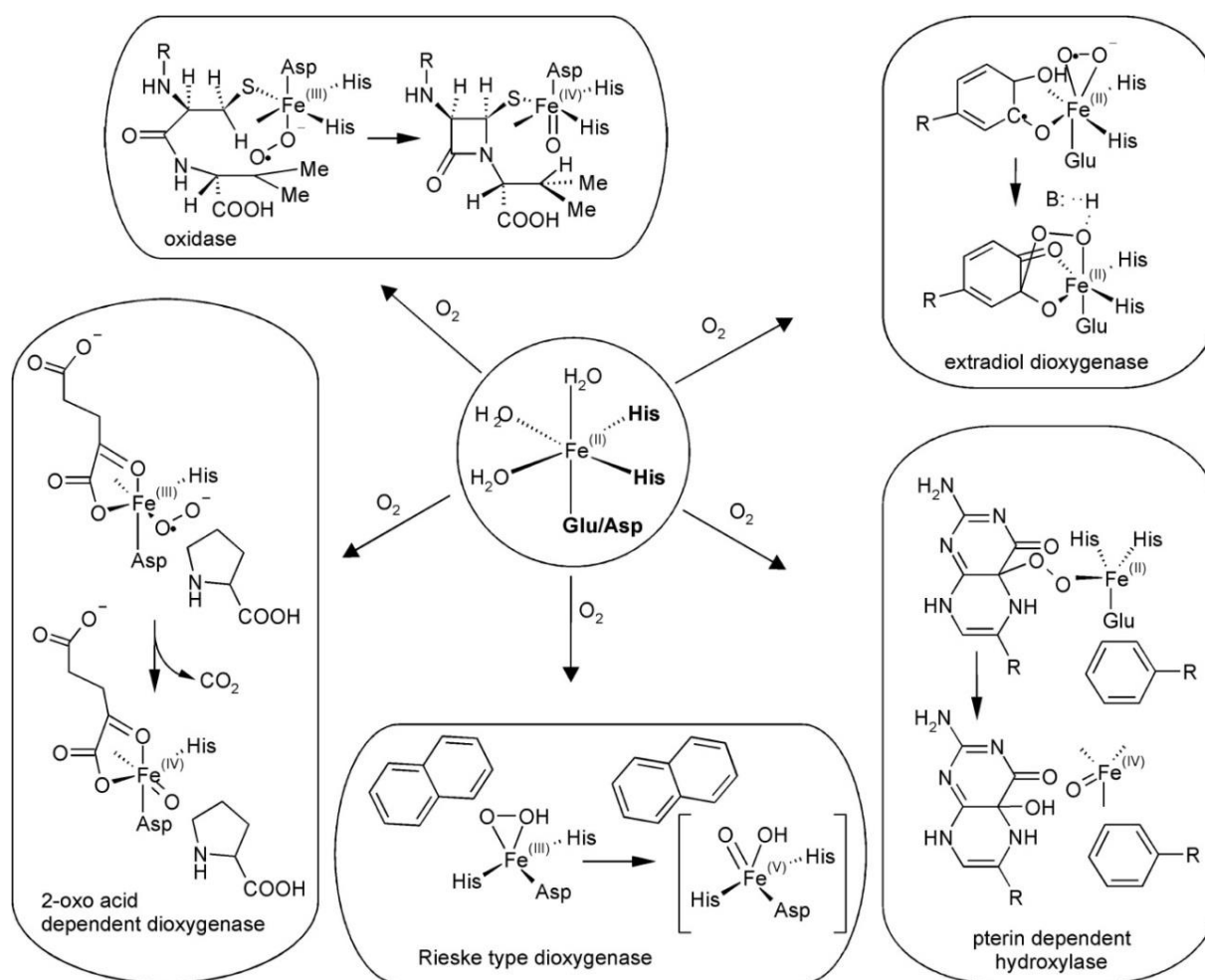
Dioxygenases incorporate two oxygen atoms into a C-C double bond generating two chiral centers and are also referred as ring hydroxylating oxygenases (RHO). These enzymes can either activate molecular oxygen (O₂) by high-spin ferrous (Fe^{II}) site, or activate

⁹⁴ [94] N. Kimura, W. Kitagawa, T. Mori, N. Nakashima, T. Tamura and Y. Kamagata, *Appl. Microbiol. Biotechnol.* **2006**, *73*, 474-484.

⁹⁵ [95] D. Gaménara, G. A. Seoane, P. Saenz-Méndez and P. D. de María, *Redox Biocatalysis: Fundamentals and Applications*, John Wiley & Sons, Inc., **2013**, p.47

⁹⁶ [96] D. T. Gibson and R. E. Parales, *Current Opinion in Biotechnology* **2000**, *11*, 236-243.

substrates by high-spin ferric (Fe^{III}) site. The O_2 activation process and the reactions with substrates diverge broadly. The reactive species formed in these reactions have been proposed to encompass 4 oxidation states of Fe and all forms of reduced O_2 as well as several of the reactive O species that derive from O-O bond cleavage. The ferric site is coordinated by a variable histidine-rich ligand environment and catalyzes intradiol aromatic ring cleavage and lipoxygenation.



Scheme 2-6 Various modes of oxygen activation by members of the **2-His//Glu** facial triad family. The triad is shown in bold in the circle. In a first reaction, a substrate or cofactor is bound and concomitantly solvent water is released to open an oxygen binding site at the iron. The three ligand sites shown to be occupied by H_2O in the central structure can be vacant, occupied by OH^- , or occupied by a weak protein ligand in different enzymes from the family. Figure adapted from Kovaleva et al.⁹⁷

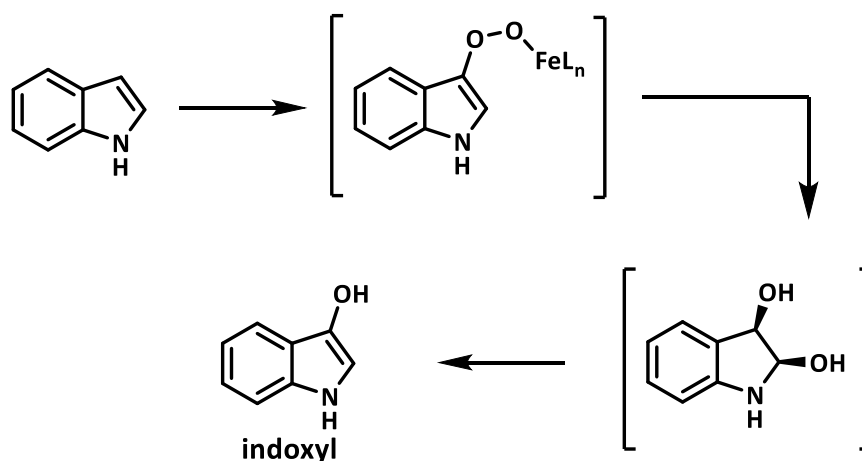
On the other hand, the ferrous site is invariably coordinated by two histidines and one aspartate or glutamate, which is a recurring motif referred to as the 2-His-1-carboxylate facial triad.⁹⁷ The remaining three ligand sites are available for the binding of substrates, cofactors, and/or O₂ during catalysis.⁹⁸ An overview of O₂ activation is shown in scheme 2-6.

There are different modes of action of RHOs reported in the literature. Due to complex nature of enzymes and multiple components involved in the transformation, there is no concrete accepted mechanism established. Although after understanding the X-ray structure of naphthalene dioxygenase^{99,100} and study of active site interactions, and with isotopic labeling, following are mechanisms reported in the literature.¹⁰¹

- i- Iron-peroxide pathway
- ii- Dioxitane pathway
- iii- 3+2 Cycloaddition pathway
- iv- Radical base mechanism

2.9.1 Iron-peroxide pathway

Evidence supporting the intermediacy of iron-bound peroxide in the naphthalene dioxygenase mediated dihydroxylation of indole was presented in 2000.¹⁰²



Scheme 2-7 iron-peroxide pathway

⁹⁷ [97] E. G. Kovaleva and J. D. Lipscomb, *Nat. Chem. Biol.* **2008**, *4*, 186-193.

⁹⁸ [98] L. M. Blank, B. E. Ebert, K. Buehler and B. Buehler, *Antioxid. Redox Signaling* **2010**, *13*, 349-394.

⁹⁹ [99] B. Kauppi, K. Lee, E. Carredano, R. E. Parales, D. T. Gibson, H. Eklund and S. Ramaswamy, *Structure* **6**, 571-586.

¹⁰⁰ [100] H. Jiang, R. E. Parales, N. A. Lynch and D. T. Gibson, *J. Bacteriol.* **1996**, *178*, 3133-3139.

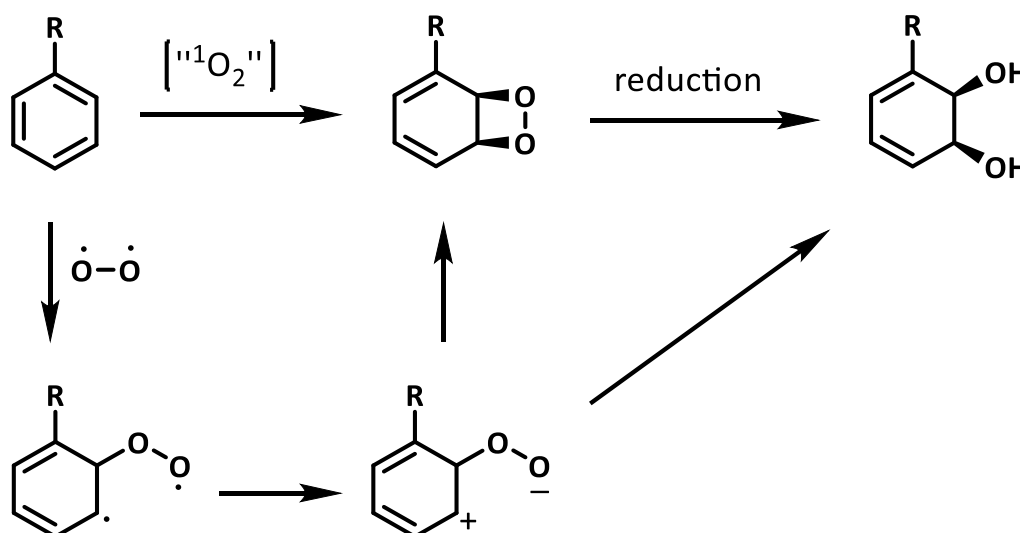
¹⁰¹ [101] T. Hudlicky and J. W. Reed, *Synlett* **2009**, 685-703.

¹⁰² [102] E. Carredano, A. Karlsson, B. Kauppi, D. Choudhury, R. E. Parales, J. V. Parales, K. Lee, D. T. Gibson, H. Eklund and S. Ramaswamy, *J. Mol. Biol.* **2000**, *296*, 701-712.

In 2003 it was revealed that an iron (II)-bound dioxygen species as a possible agent involved in the process of hydroxylation.¹⁰³

2.9.2 Dioxetane pathway

Within this process two electrons are transferred to molecular oxygen as mild oxidant, and a highly activated oxidizing agent is formed, which is stabilized by coordination to a mononuclear iron center. An interaction between oxygenase and ETC components is required for the enzyme system to transfer electrons from the electron donor to aromatic hydrocarbon electron acceptor. Within the first stage of the biooxygenation process a dioxetane bicyclic intermediate is formed, which is subsequently cleaved enzymatically to the chiral *cis*-diol product.¹⁰⁴



Scheme 2-8 Dioxetane pathway

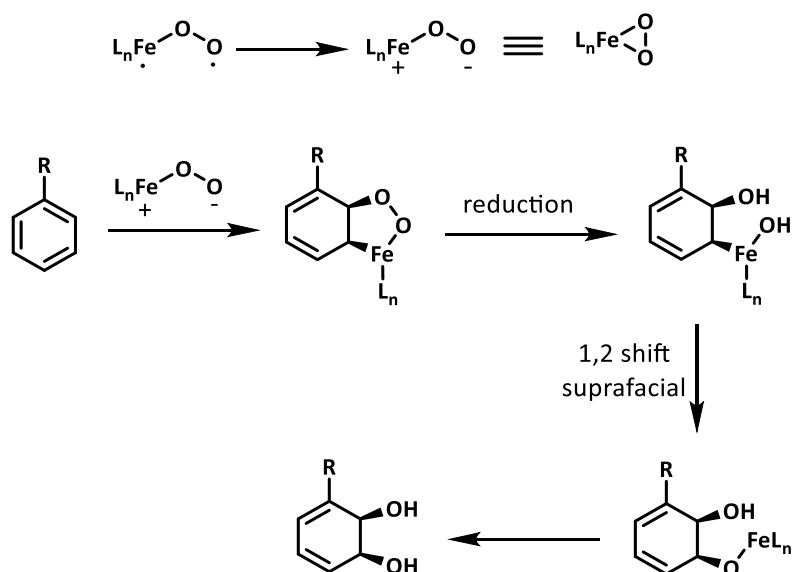
2.9.3 [3+2] cycloaddition pathway

It has been suggested that a [3+2]-type cycloaddition of iron (V) peroxide to the arene followed by reduction and a suprafacial migration of hydroxyl from the iron center is plausible as shown in scheme 2-9. Evidence supporting the intermediacy of iron-bound peroxide in the naphthalene dioxygenase-mediated dihydroxylation of indole was presented in

¹⁰³ [103] A. Karlsson, J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund and S. Ramaswamy, *Science* **2003**, 299, 1039-1042.

¹⁰⁴ [104] A. M. Jeffrey, H. J. C. Yeh, D. M. Jerina, T. R. Patel, J. F. Davey and D. T. Gibson, *Biochemistry* **1975**, 14, 575-584.

2000. Further investigations revealed an iron(II)-bound dioxygen species as a possible agent involved in the dihydroxylation.^{101,102,103}



Scheme 2-9 [3+2] cycloaddition mechanism as proposed by Parales, Karlsson¹⁰³

Boyd and Bugg reviewed different aspects of proposed mechanisms of *cis*-dihydroxylation in 2006.¹⁰⁵ On catalytic mechanism of *cis*-dihydroxylation there is no clear consensus. The involvement of [2Fe2S] iron-sulfur clusters indicates that single electron transfer events are involved in the catalytic cycle. Scheme 2-10 shows that the catalytic cycle commences with a one electron reduction of Fe(III) to Fe(II) upon substrate binding and finishes with the iron cofactor in the Fe(III) oxidation state. The observation of monooxygenase activity with certain substrates suggests that arene *cis*-dihydroxylation is not a concerted process, and that high-valent iron-oxo intermediates may be involved in monohydroxylation. It has been observed that hydrogen peroxide is released by NDO if benzene is used as substrate, which suggests that dioxygen is activated via reduction to superoxide, which in this case is uncoupled from substrate hydroxylation and is further reduced to peroxide.¹⁰⁶ Wolfe *et al.* suggested that O–O bond cleavage occurs first, to give an O=Fe(V)–OH intermediate which could effect dihydroxylation (Scheme 2-10, path A) in a fashion similar to the dihydroxylation of alkenes by OsO₄.¹⁰⁷

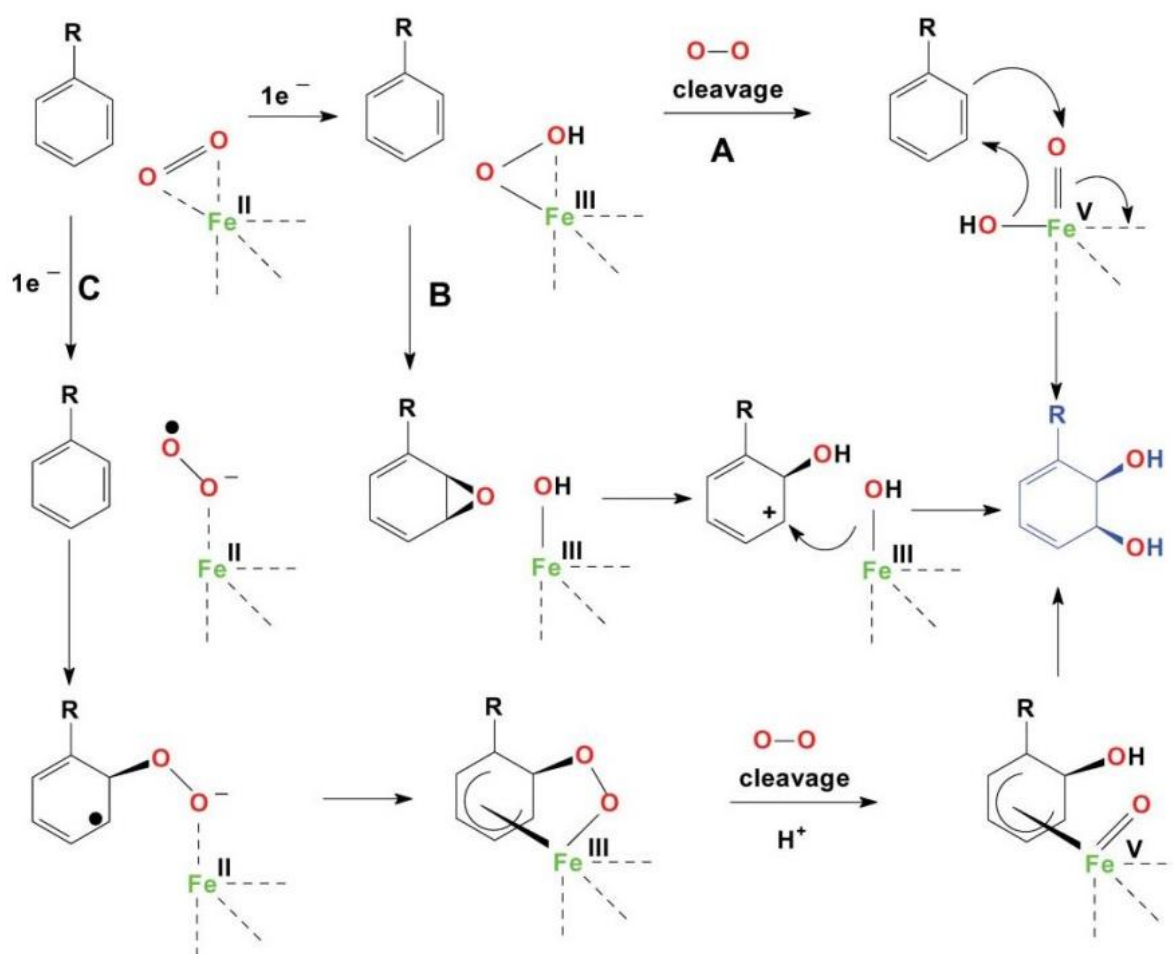
The Fe(III)–OOH moiety acting as an electrophilic oxygen species to form an epoxide (arene oxide) intermediate (Scheme 2-10, path B) has been observed to occur via lower

¹⁰⁵ [105] D. R. Boyd and T. D. H. Bugg, *Organic & Biomolecular Chemistry* **2006**, *4*, 181-192.

¹⁰⁶ [106] K. Lee, *Journal of Bacteriology* **1999**, *181*, 2719-2725.

¹⁰⁷ [107] M. D. Wolfe, J. V. Parales, D. T. Gibson and J. D. Lipscomb, *Journal of Biological Chemistry* **2001**, *276*, 1945-1953.

energy pathway. Heterolytic C–O bond cleavage of this epoxide would form a carbocation, to which the second hydroxyl group is delivered suprafacially. In case of mechanism B one might expect to observe some arene oxide byproducts (obtainable by chemoenzymatic synthesis from the corresponding *cis*-dihydrodiols)¹⁰⁸ in *cis*-dihydrodiol formation which is not the case. One would also expect electron withdrawing substrates that would destabilize a carbocation, to be strongly disfavored. The ability of Rieske dioxygenases to process electron-deficient aromatics ($R = \text{NO}_2$) would not support mechanism B.



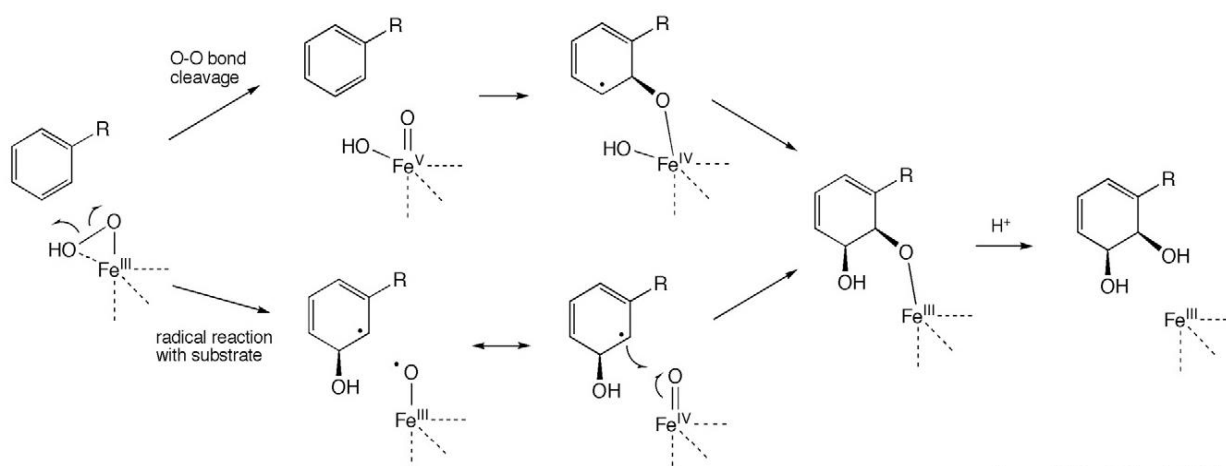
Scheme 2-10 Possible catalytic mechanisms for dioxygenase-catalysed arene *cis*-dihydroxylation¹⁰⁵

2.9.4 Free radical mechanism

Another possible mechanism involves the formation of a hydroperoxide radical intermediate followed by O–O bond cleavage and delivery of a second oxygen atom from an iron(V)-oxo species (Scheme 2-10, path C). Calculations upon this pathway revealed the

¹⁰⁸ [108] D. R. Boyd, N. D. Sharma, H. Dalton and D. A. Clarke, *Chemical Communications* **1996**, 45-46.

lower activation energy for hydroperoxide formation, but the subsequent O–O cleavage of such an intermediate was found to proceed through a high energy barrier. 2H-labelling studies on the hydroxylation of indene by NDO were found to be consistent with a substrate radical intermediate that can rotate prior to hydroxylation by an active oxygen species.¹⁰⁹ Stabilization of a substrate radical intermediate by formation of an organometallic iron species (Scheme 2-10, path C) has precedence in literature,¹¹⁰ but requires close contact of the substrate with the iron cofactor.¹¹¹ Scheme 2-11 shows possible radical-based mechanisms for cis-dihydroxylation involving an Fe^{III}-OOH intermediate, proceeding either via initial O–O bond homolysis to give an iron (V)-oxo intermediate, or reacting directly with the arene substrate to give an iron (IV)-oxo intermediate.



Scheme 2-11 Free radical mechanism proposed by Bugg involving Fe^{III}-OOH intermediate or less likely Fe(IV) intermediate¹¹¹

The mechanism of the substrate cis-dihydroxylation by dioxygenases leading to the formation of a cis-dihydrodiol was then investigated by using density functional theory (DFT) method. Nitrobenzene dioxygenase (NBDO), a member of 1,2-dioxygenase family was used to study the dihydroxylation of nitroaromatic compounds. The most likely feasible mechanism was found to be starting with the attack of the high-valent iron–oxo species on the substrate ring yielding a radical intermediate, which further evolves toward the final product.¹¹²

¹⁰⁹ [109] D. R. Boyd, N. D. Sharma, N. I. Bowers, R. Boyle, J. S. Harrison, K. Lee, T. D. H. Bugg and D. T. Gibson, *Organic & Biomolecular Chemistry* **2003**, *1*, 1298-1307.

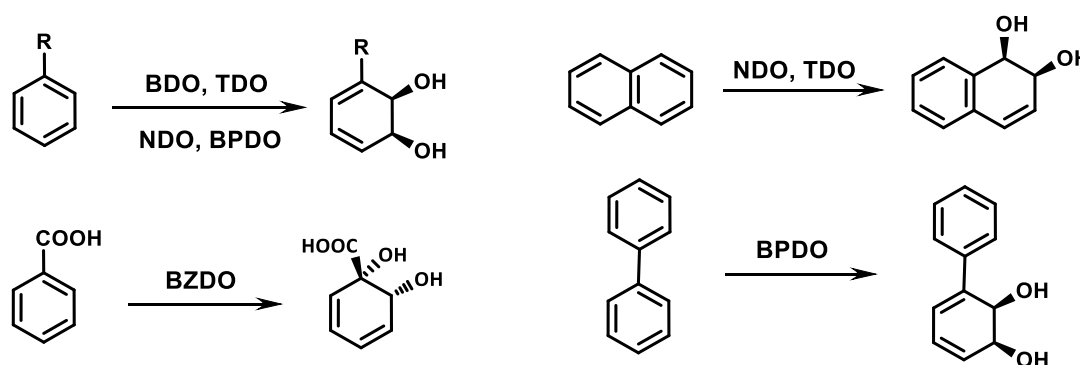
¹¹⁰ [110] A. J. Pearson, *Accounts of Chemical Research* **1980**, *13*, 463-469.

¹¹¹ [111] T. D. H. Bugg and S. Ramaswamy, *Current Opinion in Chemical Biology* **2008**, *12*, 134-140.

¹¹² [112] A. Pabis, I. Geronimo and P. Paneth, *J. Phys. Chem. B* **2014**, *118*, 3245-3256.

2.10 Regiochemistry of dioxygenases

Naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4 is known to catalyze the oxidation of more than 75 different substrates by reactions including cis-dihydroxylation, monooxygenation,^{113,114} desaturation,^{115,116} O- and N-dealkylation,¹¹⁷ and sulfoxidation.¹¹⁸ Nowadays, several dioxygenases with complementary substrate profiles have become available for synthetic applications by creation of efficient recombinant whole-cell expression systems.^{119,120,121,122}



Scheme 2-12 Regio- and stereoselectivity of dioxygenases

In particular with such delicate enzyme complexes, production of the required biocatalyst within its natural environment inside of an intact host cell (predominantly *Escherichia coli*) is highly advantageous, as this strategy enables the utilization of such catalytic entities within asymmetric synthesis with a minimum expertise in enzymology and also proteins from pathogenic organisms can be studied in a benign and safe environment.¹²³

- ¹¹³ [113] S. M. Resnick, D. S. Torok, K. Lee, J. M. Brand and D. T. Gibson, *Appl. Environ. Microbiol.* **1994**, *60*, 3323-3328.
- ¹¹⁴ [114] L. P. Wackett, L. D. Kwart and D. T. Gibson, *Biochemistry* **1988**, *27*, 1360-1367.
- ¹¹⁵ [115] D. T. Gibson, S. M. Resnick, K. Lee, J. M. Brand, D. S. Torok, L. P. Wackett, M. J. Schocken and B. E. Haigler, *J. Bacteriol.* **1995**, *177*, 2615-2621.
- ¹¹⁶ [116] D. S. Torok, S. M. Resnick, J. M. Brand, D. L. Cruden and D. T. Gibson, *J. Bacteriol.* **1995**, *177*, 5799-5805.
- ¹¹⁷ [117] S. M. Resnick and D. T. Gibson, *Biodegradation* **1993**, *4*, 195-203.
- ¹¹⁸ [118] K. Lee, J. M. Brand and D. T. Gibson, *Biochem. Biophys. Res. Commun.* **1995**, *212*, 9-15.
- ¹¹⁹ [119] G. J. Zylstra and D. T. Gibson, *Journal of Biological Chemistry* **1989**, *264*, 14940-14946.
- ¹²⁰ [120] M. J. Simon, T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W. C. Suen, D. L. Cruden, D. T. Gibson and G. J. Zylstra, *Gene* **1993**, *127*, 31-37.
- ¹²¹ [121] F. J. Mondello, *J. Bacteriol.* **1989**, *171*, 1725-1732.
- ¹²² [122] S. Yildirim, J. Zezula, T. Hudlicky, B. Witholt and A. Schmid, *Advanced Synthesis & Catalysis* **2004**, *346*, 933-942.
- ¹²³ [123] J. D. Stewart, *Current Opinion in Biotechnology* **2000**, *11*, 363-368.

Figures below gives brief overview of the possible stereochemistry,

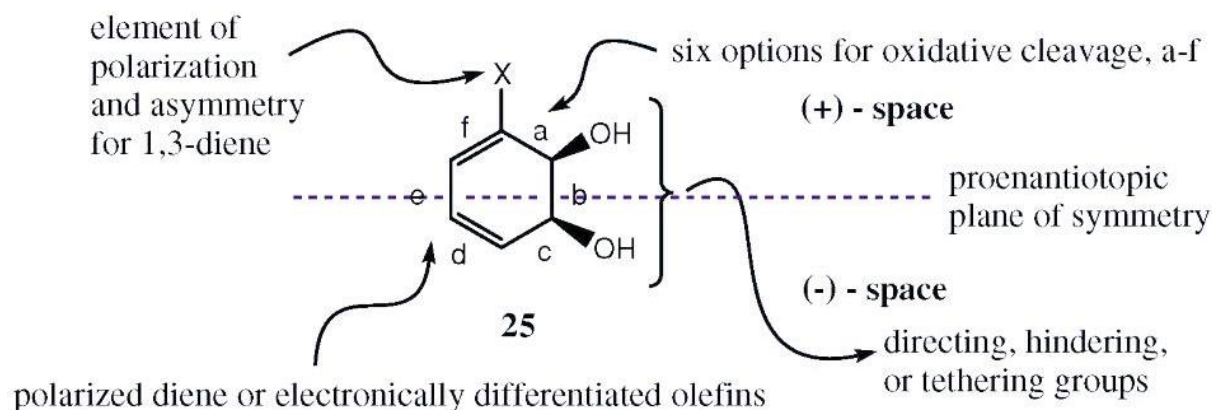


Fig. 2-10 Elements for stereomanipulation and enantiovergence in the *cis*-diene diols (+) and (-) are arbitrarily assigned spaces.¹³⁷

Figure below (Fig. 2-12) describes how the presence of functional group can affect the regiochemistry of diol formation.¹²⁴

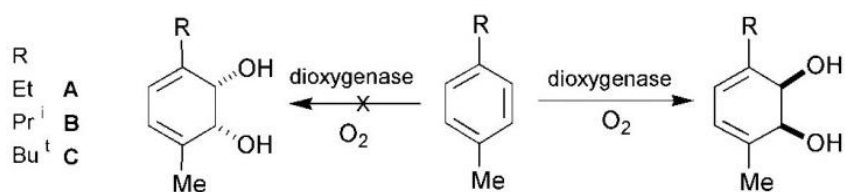
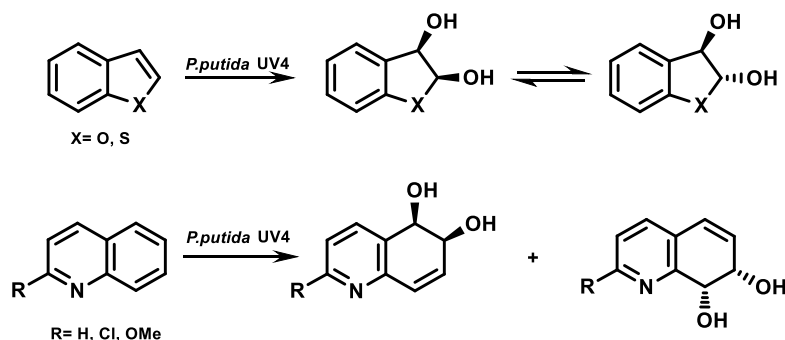


Fig. 2-11 Regiochemistry of diol formation

The most abundantly utilized type of aryl dioxygenases (2,3-dioxygenases) incorporates the additional oxygen functionalities in *ortho*- and *meta*-position to an existing substituent at the aromatic core exclusively in *cis*-configuration usually in very high optical purity. There are three different prototype enzymes distinguished by the nature of the preferably converted aromatic ring system, toluene (TDO from *Pseudomonas putida* F39/D), naphthalene (NDO, from *Pseudomonas putida* 119), and biphenyl dioxygenases (BPDO, from *Sphingomonas yanoikuyae* B8/36). The synthetic elaboration of *cis*-dioxygenated dienes has flourished in the past two decades and several total syntheses have been accomplished utilizing these chiral building blocks. Both substrate acceptance and applications in asymmetric synthesis have been comprehensively reviewed, also providing a historical perspective.

¹²⁴ [124] D. R. Boyd, N. D. Sharma, N. I. Bowers, J. Duffy, J. S. Harrison and H. Dalton, *J. Chem. Soc., Perkin Trans. 1* **2000**, 1345-1350.



Scheme 2-13 Dihydroxylation by *P. putida* UV4¹²⁴

The number of drugs incorporating a heterocyclic structural motif is legion and in the majority of cases this core system is critical for the desired biological activity. Considering this paramount role of heterocycles (in particular nitrogen containing cores) within bioactive compound synthesis, the application of DOs to access novel scaffolds has not yet received sufficient attention.

The site of dihydroxylation in heterocycles depends on the nature of the heteroaromatic system: Usually, electron rich heterocycles like thiophene are readily biooxidized but give conformationally labile products which may undergo concomitant sulfoxidation.¹²⁵ Electron deficient systems are not accepted; only pyridone derivatives give corresponding *cis*-diols. Such a differentiated behavior is also observed for benzo-fused compounds: biotransformation of benzo[*b*]thiophene gives dihydroxylation at the heterocyclic core as major product, while quinoline and other electron poor systems are oxidized at the homoaromatic core, predominantly.

2.10.1 Dioxygenation in 1,2-position (1,2-dioxygenases)

Naphthalene dioxygenases (NDO) and nitrobenzene dioxygenase (NBDO) are members of 1,2-dioxygenases. NDO is three component enzyme and belongs to 1,2-dioxygenases. Microbial 1,2-dihydroxylation of benzoic acid to produce *cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate. The evidence of benzoate dioxygenase (BZDO) expressed in *Ralstonia eutropha* B9^{126,127} (formerly known as *Alcaligenes eutrophus* 335 B9), transformation and C13 studies *Pseudomonas putida* PpU103^{128,129} (Widdowson used it in

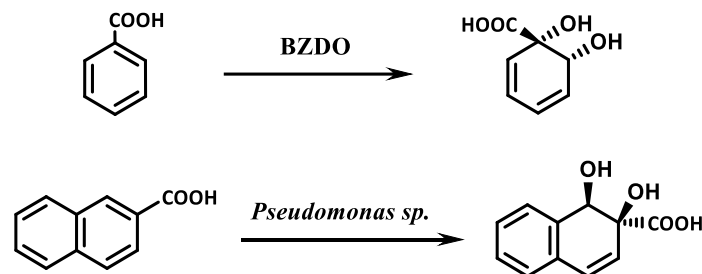
¹²⁵ [125] D. R. Boyd, N. D. Sharma, N. Gunaratne, S. A. Haughey, M. A. Kennedy, J. F. Malone, C. C. R. Allen and H. Dalton, *Organic & Biomolecular Chemistry* **2003**, *1*, 984-994.

¹²⁶ [126] A. M. Reiner and G. D. Hegeman, *Biochemistry* **1971**, *10*, 2530-2536.

¹²⁷ [127] A. G. Myers, D. R. Siegel, D. J. Buzard and M. G. Charest, *Organic Letters* **2001**, *3*, 2923-2926.

¹²⁸ [128] A. E. G. Cass, D. W. Ribbons, J. T. Rossiter and S. R. Williams, *Biochem. Soc. Trans.* **1986**, *14*, 1268-1269.

synthetic chem. and absolute configuration in 1995) *Pseudomonas putida* JT103¹³⁰ (wild type) by genetically modified *Pseudomonas putida* KTSY01 (pSYM01).¹³¹ Myers prepared lab derivatives showing all positions can be functionalized.¹³²



Scheme 2-14 Regio- and stereoselectivity of 1,2-dioxygenases

Due to high functionalization cis-diol is prone to exothermic decomposition by rearomatization. Multigram scale production and synthetic applications of cis-diol were thoroughly studied in research group of Prof. Mihovilovic by Leisch¹³³ and Thomas Fischer.¹³⁴

2.10.2 Dioxygenation in 2,3-position (2,3-dioxygenases)

Dioxygenases that incorporate two hydroxyl groups at ortho, meta (2,3-) position relative to the substituent are designated as 2,3-dioxygenases. Toluene dioxygenase (TDO), chlorobenzene dioxygenase (CDO), biphenyl dioxygenase (BPDO) are amongst the most common 2,3-dioxygenases. Unlike 1,2-dioxygenases, 2,3-dioxygenases are explored to much higher extent and there is relatively larger substrate profile available for them.

Although all 2,3-dioxygenases act the similar way in process of degradation of the substrate, there are different outcomes of the reaction depending upon number of factors. The enzyme used, the substrate itself, the electronic nature of substituent, and conditions used. TDO being more versatile or general produces all possible dihydro-diols but in different ratio. Larger substrate controls the stereochemistry of diol predominantly whereas F being the exception. Model proposed in 1993 by Boyd suggests that larger substituent affects the

¹²⁹ [129] G. N. Jenkins, D. W. Ribbons, D. A. Widdowson, A. M. Z. Slawin and D. J. Williams, *J. Chem. Soc., Perkin Trans. 1* **1995**, 2647-2655.

¹³⁰ [130] J. T. Rossiter, S. R. Williams, A. E. G. Cass and D. W. Ribbons, *Tetrahedron Lett.* **1987**, 28, 5173-5174.

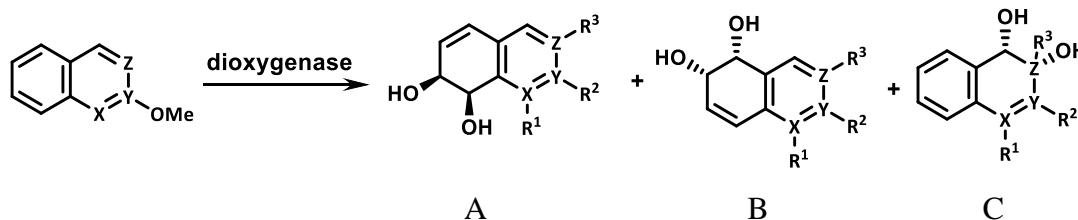
¹³¹ [131] S.-Y. Sun, X. Zhang, Q. Zhou, J.-C. Chen and G.-Q. Chen, *Applied Microbiology and Biotechnology* **2008**, 80, 977-984.

¹³² [132] M. H. Parker, B. E. Maryanoff and A. B. Reitz, *Synlett* **2004**, 12, 2095-2098.

¹³³ [133] M. D. Mihovilovic, H. G. Leisch and K. Mereiter, *Tetrahedron Letters* **2004**, 45, 7087-7090.

¹³⁴ [134] T. C. M. Fischer, H. G. Leisch and M. D. Mihovilovic, *Monatsh. Chem.* **2010**, 141, 699-707.

regiochemistry of diol formation.¹³⁵ It is also important to note that dihydroxylation on the substituted ring (Product type C) is mostly done by TDO whereas NDO, CDO, and BPDO react at the adjacent ring more predominantly. Table below shows how different microorganisms expressing different dioxygenases can produce the diols.¹³⁶



Entry 1-8: X=Y=Z=C; R¹=R³=H, R²=OMe

Entry 9: X=N, Y=Z=C; R³=H, R²=CN

Entry 10: X=N, Y=Z=C; R³=H, R²=CN

Entry 11: X=N, Y=Z=C; R³=H, R²=CN

Entry 12: Y=N, X=Z=C; R¹=H, R³=CN

Entry 13: Y=N, X=Z=C; R¹=H, R³=CN

Entry 14: X=N, Y=Z=C; R³=H, R¹=CN

Table 2-2 Possible diols formed by dioxygenases

Entry	Microorganism	Dioxy genase	Crude Yield	Diols Ratio A:B:C	Ref
1	<i>Pseudomonas putida</i> 39D	TDO	29	12:73:15	[136]
2	<i>Escherichia coli</i> JM109 (pDTG601)	TDO	64	17:69:14	[136]
3	<i>Pseudomonas putida</i> NCIB 9816	NDO	6	93:7:0	[136]
4	<i>Escherichia coli</i> JM109 (pDTG141)	NDO	57	93:7:0	[136]
5	<i>Escherichia coli</i> JM109 C534(ProR/Sac)	NDO	61	92:8:0	[136]
6	<i>Beijerinckia sp.</i> B8/36	BPDO	36	74:26:0	[136]
7	<i>Pseudomonas putida</i> UV4	TDO	46	17:52:31	[183]
8	<i>Pseudomonas fluorescens</i> N3	NDO	93	100:0:0	[181]
9	<i>Rasltonia eutropha</i> B9	TDO	10	100:0:0	c.r.
10	<i>P. putida</i> JM109 (DE3) (pDTG141)	NDO	38	100:0:0	c.r.
11	<i>P. putida</i> JM109 (PVL 1343 + PMS13)	NDO	90	100:0:0	c.r.
12	<i>P. putida</i> JM109 (DE3) (pDTG141)	NDO	25	10:90:0	c.r.
13	<i>P. putida</i> JM109 (PVL 1343 + PMS13)	NDO	52	10:90:0	c.r.
14	<i>P. putida</i> JM109 (DE3) (pDTG141)	NDO	22	60:40:0	c.r.

c.r.= current report

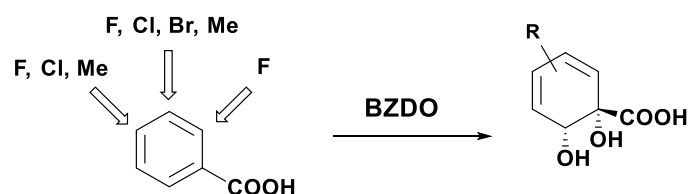
¹³⁵ [135] D. R. Boyd, N. D. Sharma, M. V. Hand, M. R. Groocock, N. A. Kerley, H. Dalton, J. Chima and G. N. Sheldrake, *Journal of the Chemical Society, Chemical Communications* **1993**, 974-976.

¹³⁶ [136] G. M. Whited, J. C. Downie, T. Hudlicky, S. P. Fearnley, T. C. Dudding, H. F. Olivo and D. Parker, *Bioorg. Med. Chem.* **1994**, 2, 727-734.

2.11 Literature precedence of biocatalytic dihydroxylations

2.11.1 Synthetic applications of dioxygenases

There are many diverse substrate subjected to dioxygenase oxidation and more than 400 products of dioxygenation have been reported. A review by Hudlicky gives a comprehensive coverage of the dioxygenase applications until 1999.¹³⁷ Recently applications of diols produced by 1,2-dioxygenases and future aspects were described by Simon E. Lewis.¹³⁸ Genetic modification of enzymes and dioxygenases in particular has widened the scope of dihydroxylations and substrates tried with engineered strains (*E. coli* JM109 pDTG601A) were discussed by Hudlicky.¹³⁹



Scheme 2-15 Potential *cis*-diols from benzoic acid

Dihydroxylated products are extremely important in many synthetic applications. Studies of the dioxygenases make it possible to consider their applications towards the synthesis of many primary and secondary synthons.^{140,141} High enantioselectivity resulting from biosynthesis of diols enables chemoenzymatic methods very useful for the enantioselective assembly of bioactive natural products.¹⁴² Figures below give brief overview of the possible reactions (Figure 2.13) of the diols.

¹³⁷ [137] T. Hudlicky, D. Gonzalez and D. T. Gibson, *Aldrichimica Acta* **1999**, 32, 35-62.

¹³⁸ [138] S. E. Lewis, *Chem. Commun.* **2014**, 50, 2821-2830.

¹³⁹ [139] J. Froese, M. A. A. Endoma-Arias and T. Hudlicky, *Org. Process Res. Dev.* **2014**, Ahead of Print.

¹⁴⁰ [140] J. A. Griffen, J. C. White, G. Kociok-Kohn, M. D. Lloyd, A. Wells, T. C. Arnot and S. E. Lewis, *Tetrahedron* **2013**, 69, 5989-5997.

¹⁴¹ [141] J. A. Griffen, S. J. Kenwright, S. Abou-Shehada, S. Wharry, T. S. Moody and S. E. Lewis, *Organic Chemistry Frontiers* **2014**, 1, 79-90.

¹⁴² [142] K. A. B. Austin, M. Matveenko, T. A. Reekie and M. G. Banwell, *Chem. Aust.* **2008**, 75, 3-4, 6-7.

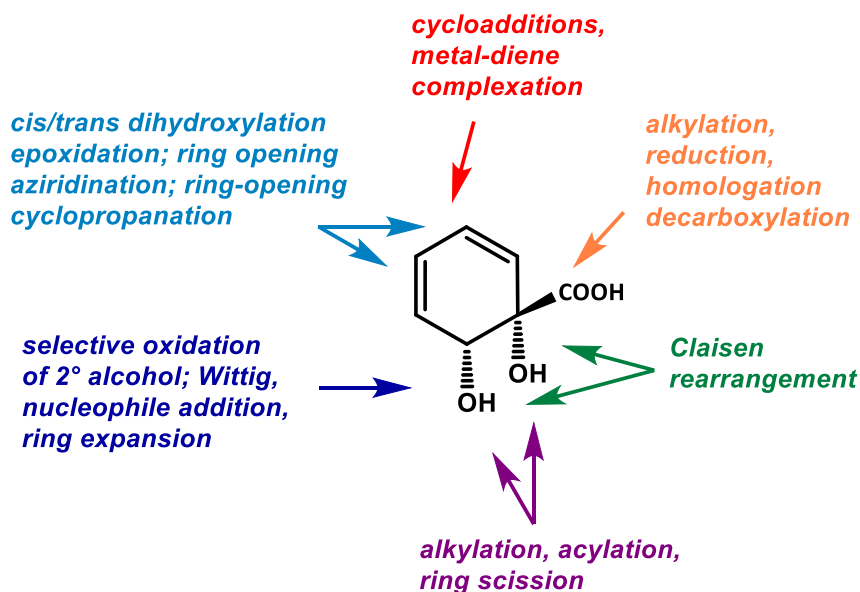


Fig. 2-12 Possible applications of 1,2-diol, adapted from Griffin¹⁴⁰

Many useful secondary synthons were synthesized by Banwell starting from 2,3-diols of halobenzenes.^{143,144} Ethylbenzoate was used as substrate for 2,3-dioxygenase in the synthesis of Oseltamivir (Tamiflu).^{145,146,147} The use of substituted cyclohexadienediols as versatile chiral synthons was reported in a review by Widdowson.¹⁴⁸ Diols produced by biocatalytic action of dioxygenases have been used in formal total synthesis of a number of compounds. Chemoenzymatic formal synthesis of (-)- and (+)-epibatidine was reported by Griffin in 2013.¹⁴⁹ Starting from benzoic acid and 1,2-dioxygenases from *Alcaligenes eutrophus* B9 getting cis-diol on 13 grams per liter scale, Myers group prepared tetracycline derivatives.^{150,151} Formal total synthesis of Platencin was made possible starting from cis-diol prepared from dioxygenase dihydroxylation of iodobenzene.¹⁵² Total synthesis of Codeine,

¹⁴³ [143] D. M. Pinkerton, M. G. Banwell and A. C. Willis, *Aust. J. Chem.* **2009**, *62*, 1639-1645.

¹⁴⁴ [144] D. M. Pinkerton, M. G. Banwell and A. C. Willis, *Organic Letters* **2009**, *11*, 4290-4293.

¹⁴⁵ [145] T. Hudlicky in *Processes and intermediates for the preparation of oseltamivir and analogs thereof for treatment of influenza*, Vol. (Ed. P. I. Appl), Brock University, Can., **2009**, p. 106pp.

¹⁴⁶ [146] B. Sullivan, I. Carrera, M. Drouin and T. Hudlicky, *Angewandte Chemie, International Edition* **2009**, *48*, 4229-4231.

¹⁴⁷ [147] L. Werner, A. Machara, B. Sullivan, I. Carrera, M. Moser, D. R. Adams and T. Hudlicky, *J. Org. Chem.* **2011**, *76*, 10050-10067.

¹⁴⁸ [148] D. A. Widdowson, D. W. Ribbons and S. D. Thomas, *Janssen Chim. Acta* **1990**, *8*, 3-9.

¹⁴⁹ [149] D. R. Boyd, N. D. Sharma, M. Kaik, P. B. A. Mc Intyre, P. J. Stevenson and C. C. R. Allen, *Organic & Biomolecular Chemistry* **2012**, *10*, 2774-2779.

¹⁵⁰ [150] M. G. Charest, C. D. Lerner, J. D. Brubaker, D. R. Siegel and A. G. Myers, *Science* **2005**, *308*, 395-398.

¹⁵¹ [151] M. G. Charest, D. R. Siegel and A. G. Myers, *Journal of the American Chemical Society* **2005**, *127*, 8292-8293.

¹⁵² [152] K. A. B. Austin, M. G. Banwell and A. C. Willis, *Organic Letters* **2008**, *10*, 4465-4468.

Balanol, Pancratistatin, and Oseltamivir involving dioxygenases was performed by Hudlicky.¹⁵³

2.11.2 *cis*-Dihydrodiols as synthetic intermediates

A number of bioactive natural compounds have *cis*-diol motif in their scaffold. A number of diols produced biocatalytically are now available on commercial scale. With increase of number of diols commercially available and possibility to synthesize them with basic skills involved, makes it very useful tool for intermediate synthesis. Few selected examples of intermediate synthesis are selected from literature.^{154,155,156}

2.11.3 Dihydroxylation of monocyclic compounds

Dihydroxylation of aromatics including halobenzoates, *b*-bromoethylbenzene,¹⁵⁷ 1-phenyl-2-acetoxyethane¹⁵⁸ phenols and biphenyls¹⁵⁹ and regioisomeric 1,2- and 3,4-*cis*-dihydrodiols.¹⁶⁰ Hamdy gave some insight of potential pharmaceutical compounds from dioxygenase-derived chiral metabolites.¹⁶¹

2.11.4 Dihydroxylation of polycyclic compounds

Few selected examples of enzymatic dihydroxylation of polycyclic aromatic compounds include tricyclic azaarenes in synthesis of furoquinoline alkaloids¹⁶² anthracene,^{163,164,165} and benzanthracene.^{166,167} Method of enzymatic dihydroxylation has been also employed for the detection of PAHs in food.¹⁶⁸

¹⁵³ [153] T. Hudlicky, *Pure Appl. Chem.* **2010**, 82, 1785-1796.

¹⁵⁴ [154] R. A. Johnson in *Microbial Arene Oxidations*, Vol. Organic Reactions, John Wiley & Sons, Inc., **2004**.

¹⁵⁵ [155] D. J. Y. D. Bon, B. Lee, M. G. Banwell and I. A. Cade, *Chim. Oggi* **2012**, 30, 22-27.

¹⁵⁶ [156] D. R. Boyd, N. D. Sharma, B. Byrne, M. V. Hand, J. F. Malone, G. N. Sheldrake, J. Blacker and H. Dalton, *J. Chem. Soc., Perkin Trans. 1* **1998**, 1935-1944.

¹⁵⁷ [157] V. Varghese and T. Hudlicky, *Angew. Chem., Int. Edit.* **2014**, 53, 4355-4358.

¹⁵⁸ [158] M. A. A. Endoma-Arias, J. R. Hudlicky, R. Simionescu and T. Hudlicky, *Advanced Synthesis & Catalysis* **2014**, 356, 333-339.

¹⁵⁹ [159] D. R. Boyd, N. D. Sharma, P. J. Stevenson, M. Blain, C. McRoberts, J. T. G. Hamilton, J. M. Argudo, H. Mundi, L. A. Kulakov and C. C. R. Allen, *Organic & Biomolecular Chemistry* **2011**, 9, 1479-1490.

¹⁶⁰ [160] D. R. Boyd, N. D. Sharma, N. M. Llamas, C. R. O'Dowd and C. C. R. Allen, *Organic & Biomolecular Chemistry* **2007**, 5, 2267-2273.

¹⁶¹ [161] H. A. Hassan, *Curr. Trends Biotechnol. Pharm.* **2012**, 6, 389-397.

¹⁶² [162] D. R. Boyd, N. D. Sharma, J. G. Carroll, P. L. Loke, C. R. O'Dowd and C. C. R. Allen, *RSC Advances* **2013**, 3, 10944-10955.

¹⁶³ [163] L. Schuler, Y. Jouanneau, S. M. Ni Chadhain, C. Meyer, M. Pouli, G. J. Zylstra, P. Hols and S. N. Agathos, *Appl. Microbiol. Biotechnol.* **2009**, 83, 465-475.

2.11.5 Industrial applications of biooxidations

Many enzymes can catalyze not only their natural reaction with their natural substrate, but also accept a variety of non-natural substrates and provide a relatively broad spectrum of reaction possibilities. Keeping in mind the industrial applications of these enzymes, acceptance of broad spectrum of substrates and conditions leading to several reactivities and formation of different products can be very useful. A correct and rational understanding of enzymatic profile can facilitate the development of novel processes based on same biocatalyst. Biocatalytic reactions have become the subject of first choice from reactions of mere lab curiosity.

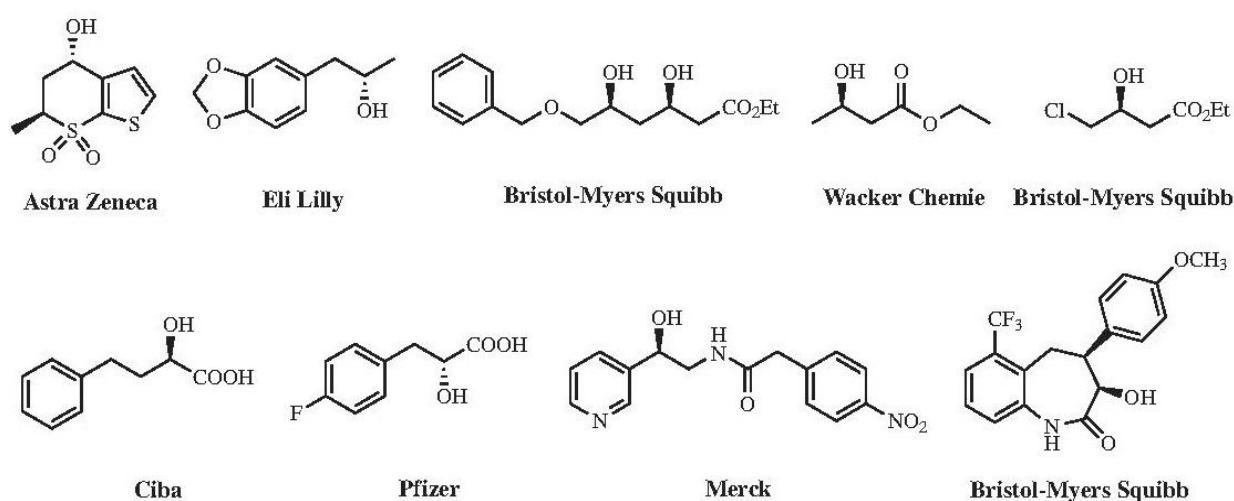


Fig. 2-13 Selected examples of compounds produced involving enzyme-catalyzed redox processes⁹⁵

Few strategies involved for the development of industrial processes include cloning and overexpression of biocatalysts, genetic improvement of enzymes metagenomics, biocatalytic promiscuity, cofactor regeneration strategies, and biphasic system for higher

¹⁶⁴ [164] D. M. Jerina, H. Selander, H. Yagi, M. C. Wells, J. F. Davey, V. Mahadevan and D. T. Gibson, *J. Am. Chem. Soc.* **1976**, 98, 5988-5996.

¹⁶⁵ [165] M. N. Akhtar, D. R. Boyd, N. J. Thompson, M. Koreeda, D. T. Gibson, V. Mahadevan and D. M. Jerina, *J. Chem. Soc., Perkin Trans. 1* **1975**, 2506-2511.

¹⁶⁶ [166] D. T. Gibson, V. Mahadevan, D. M. Jerina, H. Yagi and H. J. C. Yeh, *Science* **1975**, 189, 295-297.

¹⁶⁷ [167] D. M. Jerina, P. J. Van Bladeren, H. Yagi, D. T. Gibson, V. Mahadevan, A. S. Neese, M. Koreeda, N. D. Sharma and D. R. Boyd, *Journal of Organic Chemistry* **1984**, 49, 3621-3628.

¹⁶⁸ [168] J. F. Krebs, J. Duvall-Jisha and S. Resnick in *Rapid detection of petroleum in food*, Vol. Bio Scientific Corporation, USA . **2014**, p. 11.

reaction yields. Chemoenzymatic synthesis of *cis,cis*-muconic acid starting from benzoic acid was reported in 2006.¹⁶⁹

2.11.6 Dihydroxylation of heteroaromatic compounds

NPAHs dihydroxylations are in general low yielding biotransformations as compared to naphthalenes. This could be due to the solubility of the substrates in biotransformation media and relative inability to effectively transport the substrate across the growing cells membrane. Another limiting factor is the toxicity of substrate itself for the growing cells to efficiently metabolize the NPAHs while maintaining the dioxygenase production. Aromatic heterocycles quinoline, isoquinoline, azaarenes have been biotransformed to find synthetic applications.^{170, 171}

Biotransformation of quinolines with dioxygenases results in dihydroxylation on both carbo- and heterocyclic rings to form diols.¹⁷² Aromatic nitriles are particularly important substrates for dihydroxylation due to number of possible reactions that could be carried out with the nitrile groups including oxidations and reductions. Carboxylic acids are reported to be poor or no substrates for dioxygenases. There are few examples in the literature where dioxygenase oxidation can be combined with selective nitrilase hydrolysis in order to prepare chiral carboxylic acids.¹⁷³

2.11.7 Biomimetic *cis*-dihydroxylations

Rieske dioxygenase enzymes contain in their active site a mononuclear iron center coordinated to a 2-histidine-1-carboxylate motif.¹⁷⁴ There have been few attempts made to study the active site of dioxygenase and synthesize a catalyst to perform the reaction. The group of Que and coworkers was the first to synthesize a successful catalyst based on the active site of NDO.¹⁷⁵ In order to mimic the facial triad of dioxygenase, a Fe(II) complex with

¹⁶⁹ [169] A. Liese, K. Seelbach, C. Wandrey and Editors, *Industrial Biotransformations; Second, Completely Revised and Extended Edition*, Wiley-VCH **2006**, p. 556 pp.

¹⁷⁰ [170] D. R. Boyd, N. D. Sharma, M. R. J. Dorrity, M. V. Hand, R. A. S. McMordie, J. F. Malone, H. P. Porter, H. Dalton, J. Chima and G. N. Sheldrake, *J. Chem. Soc., Perkin Trans. 1* **1993**, 1065-1071.

¹⁷¹ [171] D. R. Boyd, R. A. S. McMordie, H. P. Porter, H. Dalton, R. O. Jenkins and O. W. Howarth, *J. Chem. Soc., Chem. Commun.* **1987**, 1722-1724.

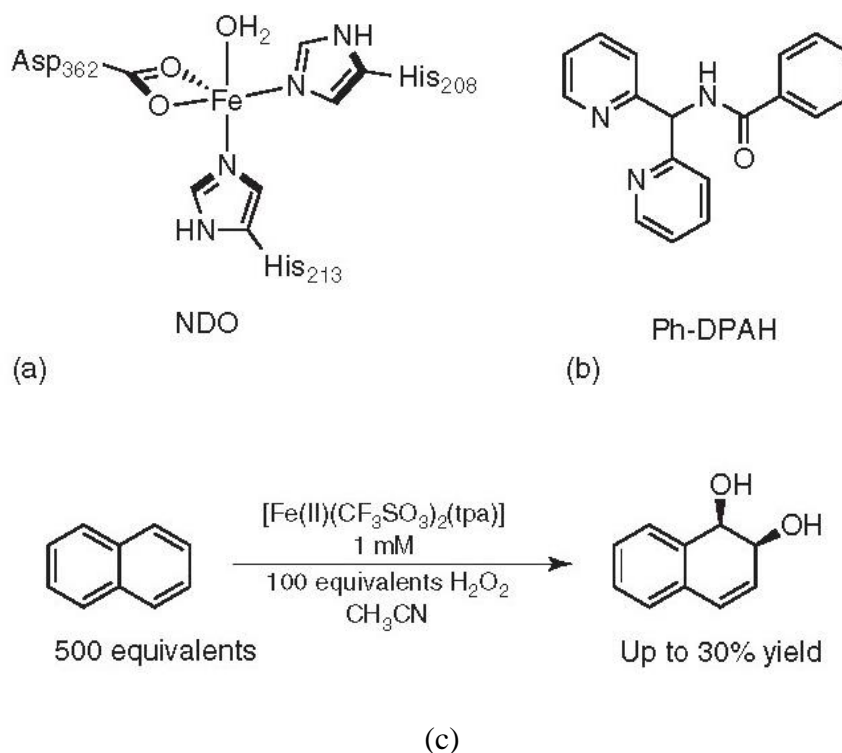
¹⁷² [172] D. R. Boyd, N. D. Sharma, J. G. Carroll, J. F. Malone, D. G. Mackerracher and C. C. R. Allen, *Chemical Communications* **1998**, 683-684.

¹⁷³ [173] S. Yildirim, R. Ruinatscha, R. Gross, R. Wohlgemuth, H.-P. E. Kohler, B. Witholt and A. Schmid, *Journal of Molecular Catalysis B: Enzymatic* **2006**, 38, 76-83.

¹⁷⁴ [174] P. C. A. Bruijninx, G. van Koten and R. J. M. Klein Gebbink, *Chemical Society Reviews* **2008**, 37, 2716-2744.

¹⁷⁵ [175] K. Chen and J. L. Que, *Angewandte Chemie International Edition* **1999**, 38, 2227-2229.

a N,N,O-donor set ligand Ph-DPAH was prepared as synthetic model. This catalyst performs the oxidation of various alkenes with excellent selectivity towards *cis*-dihydroxylation.¹⁷⁶ Along the same line, in a recent work, the authors described several structurally related complexes that share the same facial N,N,O triad. All of these catalysts exhibit a high selectivity toward *cis*-dihydroxylation in their alkene oxidation reactions.



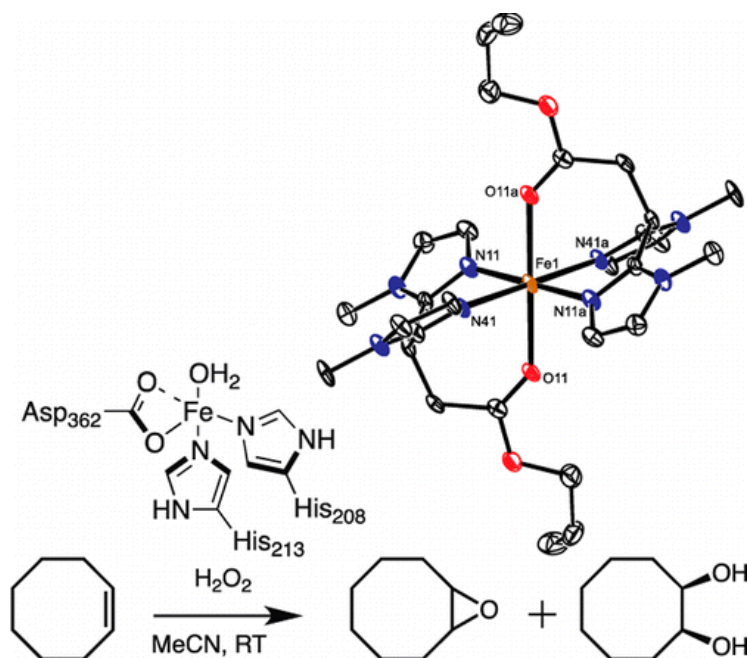
Scheme 2-16 Bioinspired catalysts- (a) Active site of Rieske oxygenase enzyme, NDO. (b) Ligand Ph-DPAH used as a mimic of Rieske oxygenases active center as described by Que. (c) A synthetic functional model of NDO.

Naphthalene 1,2-dioxygenase (NDO) mediates the *cis*-dihydroxylation of an arene. Such reaction lacks any precedent in synthetic chemistry until a recent report described that $[\text{Fe}(\text{II})(\text{CF}_3\text{SO}_3)_2(\text{tpa})]$ catalyzes the oxidation of naphthalene with H_2O_2 , affording four oxidation products, *cis*-1,2-dihydro-1,2-naphthalenediol, 1-naphthol, 2-naphthol, and 1,4-naphthoquinone. The diol was obtained as a major product and it constitutes the metabolite in NDO enzymes. As previously indicated, the key limitation of these systems is that a relatively low ton and substrate conversion are obtained. Exceptional catalysts that overcome these limitations have started to appear recently.¹⁷⁷

¹⁷⁶ [176] P. D. Oldenburg, A. A. Shteinman and L. Que, *Journal of the American Chemical Society* **2005**, *127*, 15672-15673.

¹⁷⁷ [177] Y. Feng, C.-y. Ke, G. Xue and L. Que, *Chemical Communications* **2009**, 50-52.

Bioinspired mononuclear Fe(II) triflate complexes derived from an extended series of N,N,O-Ligated 3,3- bis(1-alkylimidazole-2-yl)propionate ester (BAIP) ligand scaffold were reported to incorporate the epoxidation/cis-dihydroxylation of cyclooctene with H₂O₂.¹⁷⁸



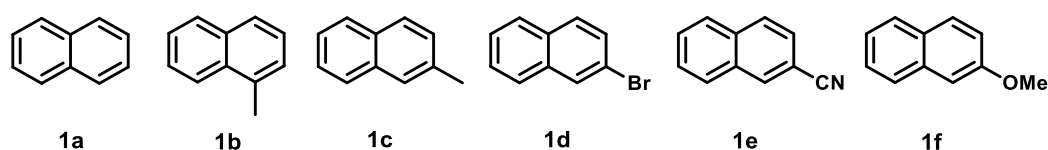
Scheme 2-17 Bioinspired catalysts based on iron triflate complexes¹⁷⁸

¹⁷⁸ [178] M. A. H. Moelands, S. Nijse, E. Folkertsma, B. de Bruin, M. Lutz, A. L. Spek and R. J. M. Klein Gebbink, *Inorganic Chemistry* **2013**, 52, 7394-7410.

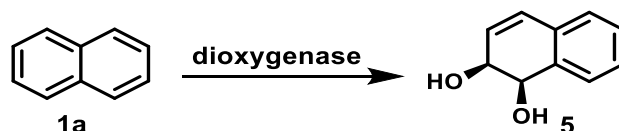
2.12 Literature precedence for selected substrates

A brief summary of literature precedence of PAHs and NPAHs biotransformations with dioxygenases is given below. It includes literature about selected substrates from naphthalene, quinoline, isoquinoline, and mono substituted counterparts.

2.12.1 Naphthalenes



Naphthalene (**1a**) was reported as one of the earliest substrates for the testing and modification of dioxygenases. It has been studied extensively and used as reference substrate for a number of dioxygenase reactions. It is also used as carbon source for the growth to perform some whole cell mediated biotransformations involving naphthalene dioxygenase (NDO).¹⁸³



Naphthalene diol **5** is not stable under room temperature and tends to rearomatize by elimination of water. It was stored at -30°C in order to avoid decomposition.

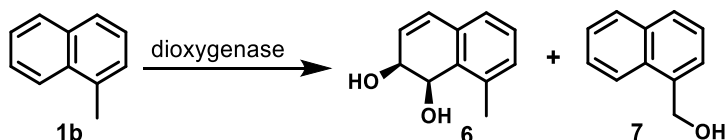
1-Methylnaphthalene (**1b**) was first reported as substrate by Hudlicky¹⁷⁹ and later by K. Shindo¹⁸⁰ with details. The aliphatic group also undergoes side chain oxidation to alcohols or even to carboxylic acids.¹⁸¹ Monooxygenation on methyl group on substituted PAHs was reported to occur with NDO and NBDO.¹⁸²

¹⁷⁹ [179] M. E. Deluca and T. Hudlicky, *Tetrahedron Letters* **1990**, *31*, 13-16.

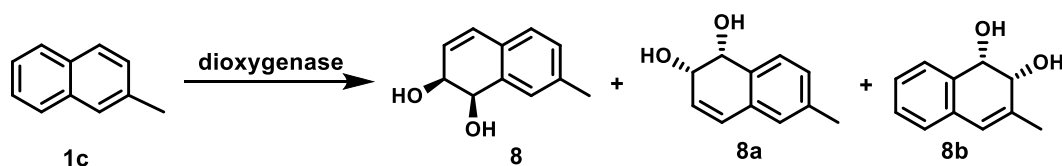
¹⁸⁰ [180] K. Shindo, A. Osawa, Y. Kasai, N. Iba, A. Saotome and N. Misawa, *Journal of Molecular Catalysis B: Enzymatic* **2007**, *48*, 77-83.

¹⁸¹ [181] G. Bestetti, D. Bianchi, A. Bosetti, P. Gennaro, E. Galli, B. Leoni, F. Pelizzoni and G. Sello, *Applied Microbiology and Biotechnology* **1995**, *44*, 306-313.

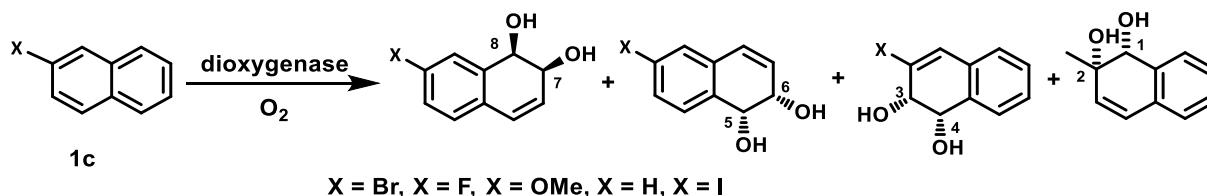
¹⁸² [182] A. Toendervik, P. Bruheim, L. Berg, T. E. Ellingsen, H. K. Kotlar, S. Valla and M. Throne-Holst, *J. Biosci. Bioeng.* **2012**, *113*, 173-178.



2-Substituted naphthalenes including 2-methylnaphthalene **1c** were studied in details by Boyd *et al.*,⁸¹ Hudlicky *et al.*,¹⁷⁹ Bestetti *et al.*,¹⁸¹ and Marcin Kwit *et al.*¹⁸³ 2-Methyl naphthalene produced all three possible products of *cis*-dihydroxylation at substituted ring (3,4-diol) and non-substituted ring (5,6 and 7,8 diol). Most favored dioxxygenation site being 7,8-position of 2-methylnaphthalene (yields upto 250 mg L⁻¹) and least favored being 5,6-position (yields upto 10%).



Substituted naphthalene substrates on 2-position with substituents including F-, Br-, -OMe, -CN, -Me, and -I, were reported by Kwitt and Boyd in 2008.¹⁸³ Naphthalene-2-carbonitrile (**3d**) was also reported as substrate and according to the literature¹⁸³ all three possible products of dihydroxylation (7-8 diol = 15%, 5,6-diol = 57%, 3,4-diol = 28%) were reported to be formed by using *Pseudomonas putida* expressing TDO enzyme.

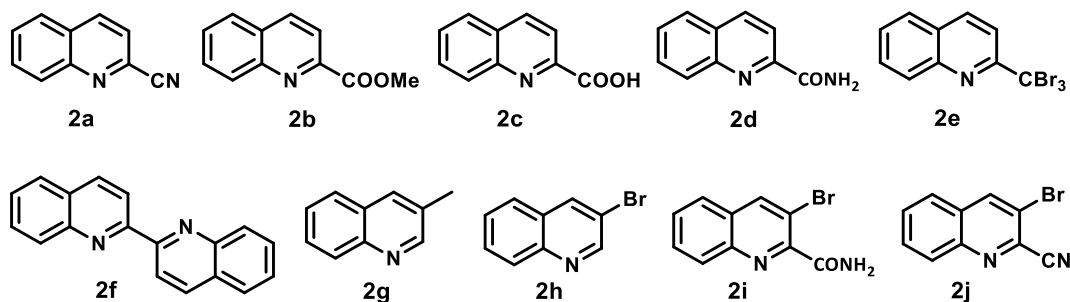


Scheme 2-18 Possible *cis*-dihydrodiol regioisomers formed as a result of dioxxygenase-catalyzed *cis*-dihydroxylation of monosubstituted naphthalene.¹⁸³

Our major interest in dihydroxylation of substituted naphthalene was to study the substrate acceptance behavior under our set conditions and also to obtain the isolated diols in order to study the cyclic carbonate formation behavior as discussed in section 3.7 and 4.8.

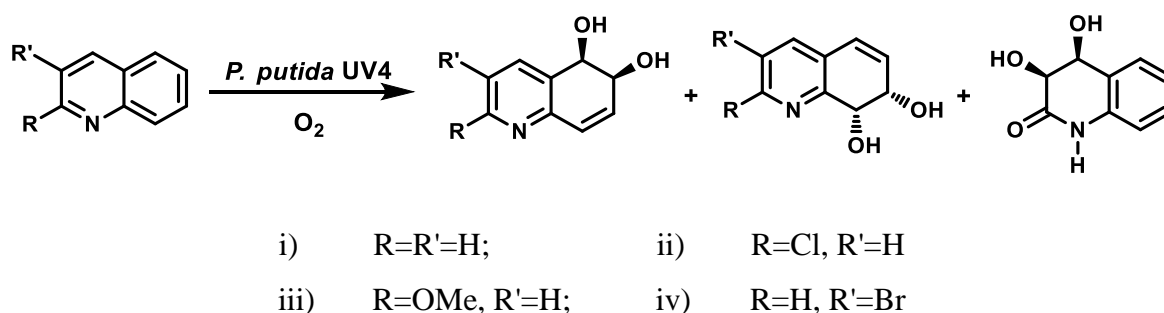
¹⁸³ [183] M. Kwit, J. Gawronski, D. R. Boyd, N. D. Sharma, M. Kaik, R. A. More O'Ferrall and J. S. Kudavalli, *Chemistry--A European Journal* **2008**, *14*, 11500-11511.

2.12.2 Quinolines



There is vast diversity of substrates acceptance and relative ratio of preferred diol formation with the substituted NPAHs. It depends on number of factors including type of recombinant system, position, size, and polarity of substituent.

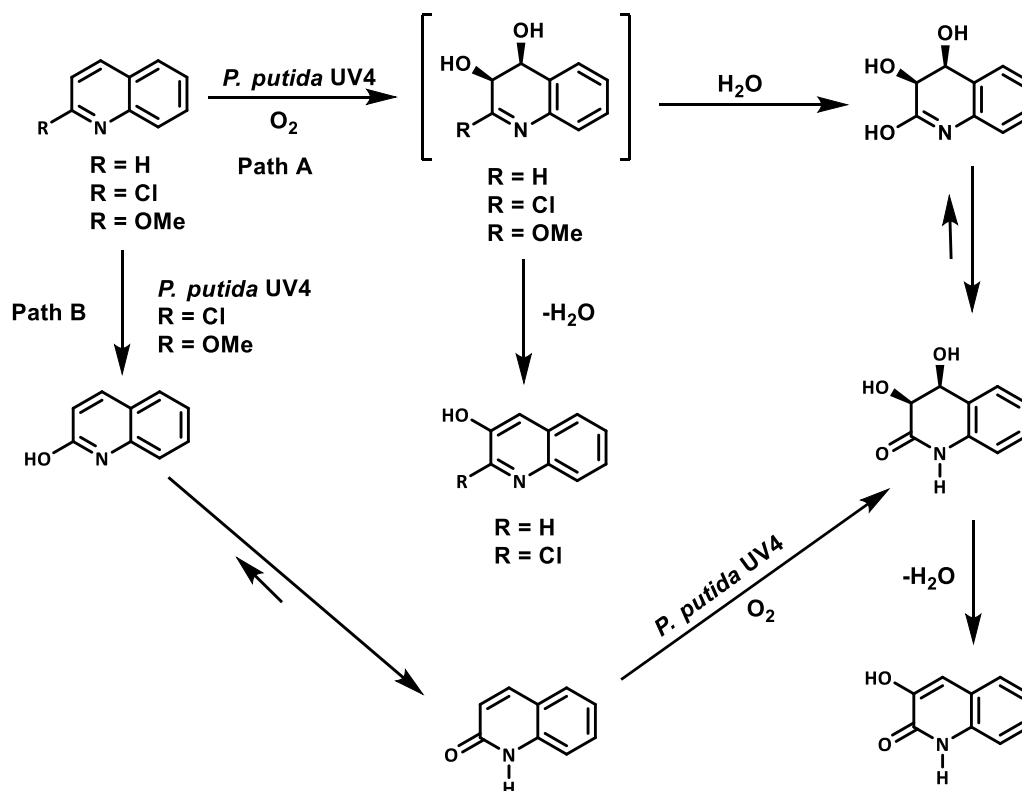
Another less relevant example would be that of 3-bromoquinoline (**2b**) (and section 3.5.8) giving 5,6-diol as the exclusive product of dihydroxylation. Earlier studies on NPAHs have shown the diols could be formed by *cis*-dihydroxylation at 5,6-bond and 7,8-bond of the carbocyclic ring. Unsubstituted quinolines and isoquinolines are reported to produce all three possible diol.¹⁸⁴



Scheme 2-19 *cis*-Dihydrodiol metabolites isolated by biotransformation of substituted quinolines.¹⁸⁴

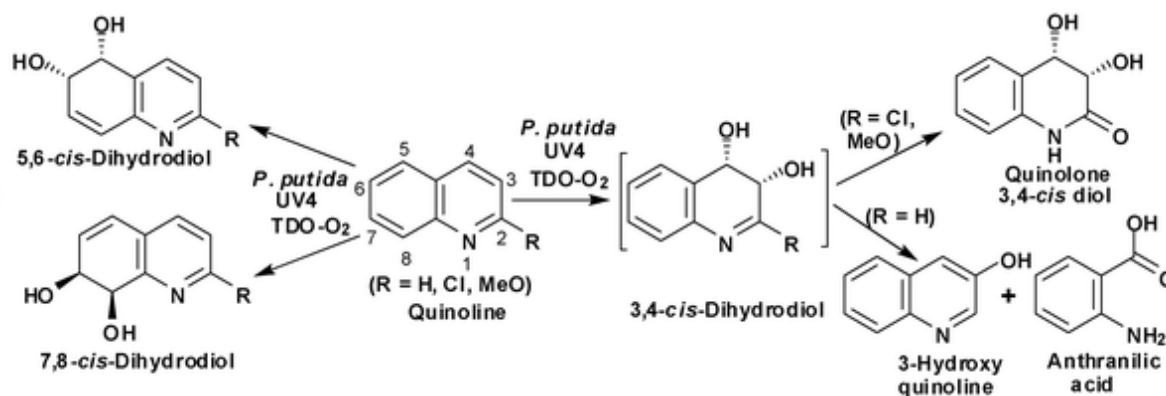
Dihydroxylation was also found to occur at 3,4-bond of heterocyclic ring resulting in rather unstable diols which decomposed resulting in formation of mono-hydroxy products by the elimination of water. Dihydroxylation of the 3,4-bond in the electron-deficient pyridine ring of the quinoline substrates was found to yield only minor metabolites in comparison with its carbocyclic 5,6-and 7,8-bonds. The formation of (3*S*, 4*S*)-3,4-dihydroxy-3,4-dihydro-2-quinolone could be due to the hydrolysis of unstable 3,4-*cis* diol formed by dihydroxylation on the heterocyclic ring.¹⁸⁴

¹⁸⁴ [184] D. R. Boyd, N. D. Sharma, L. V. Modyanova, J. G. Carroll, J. F. Malone, C. C. R. Allen, J. T. G. Hamilton, D. T. Gibson, R. E. Parales and H. Dalton, *Can. J. Chem.* **2002**, *80*, 589-600.



Scheme 2-20 Schematic representation of 3,4-dihydroxylation at the pyridine ring and formation of other metabolites from the product.¹⁸⁴

The 3-hydroxyquinoline and anthranilic acid metabolites of quinoline were assumed to be derived from the undetected heterocyclic cis-3,4-dihydrodiol intermediate.¹⁶²



Scheme 2-21 Further evidence of 3,4-dihydroxylation as seen by the isolation of anthranilic acid metabolites of quinoline¹⁶²

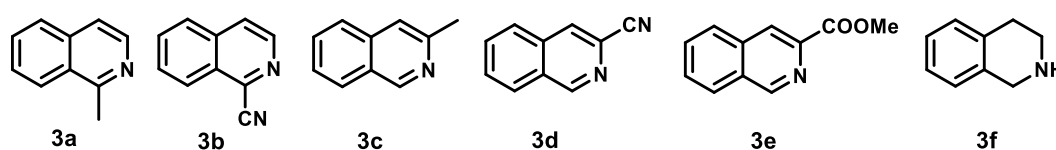
There are also few examples in the literature representing monohydroxylation of the alkyl group regardless of its position on carbocyclic or heterocyclic ring. Monohydroxylation of alkyl substituent is kind of competing reaction with the dihydrodiol formation as alkyl side chain hydroxylated metabolites generally stay in the aqueous phase without undergoing

further dihydroxylation and forming triols or tertaols.¹⁸⁵ So far there are examples of cis-diol formation of monosubstituted azaarenes where as there are very few examples of diols formed by cis-dihydroxylation of polysubstituted azaarenes. This might be due to different electronic effect of substituents and active site of enzyme. Monocyclic aromatic compounds with two substituents are reported to be the substrates for dihydroxylation.¹⁸⁶

3-Bromoquinoline (**2b**) was first reported by Boyd et al in 2002¹⁸⁴ as accepted substrate for TDO using recombinant whole-cell mediated biotransformation with *P. putida* UV4. It was biotransformed on 10L scale with 7.5g of substrate and giving 23% 5,6-diol as exclusive product.¹⁸⁴

As indicated from previous examples, PAHs and heteroaromatics with polycyclic rings are moderately accepted substrates for 2,3-dioxygenases. Benzoic acid and derivatives are reported to be accepted substrates dioxygenases. There have been well documented studies in syntheses of *cis*-dihydrodiol metabolites of benzoic acid derivatives with 1,2-dioxygenases and 2,3-dioxygenases and their application in various fields. T. Fischer from research group of Prof. Mihovilovic had studied multi gram scale dihydroxylation of benzoic acid with *Ralstonia eutropha* B9 and precipitating the resulting diol as sodium salt that was subsequently used as starting material for the total synthesis of Piperenol B and Uvarirufol A.^{134,187}

2.12.3 Isoquinolines



Biotransformation of isoquinoline was reported by Boyd in 1993 by *P. putida* and 5,6-dihydrodiol along with 1-, 4-, 5-, and 8- hydroxyl isoquinolines were isolated. The identification of monohydroxy isoquinolines was evidence of *cis*-dihydroxylation of the substrate and subsequent dehydration of corresponding diol in order to restore the aromaticity.¹⁷⁰ Literature evidence of somewhat related substrate 3-methylisoquinoline was reported to produce corresponding 7,8-diol (73%) and 3-methylisoquinoline-5-ol (27%) with

¹⁸⁵ [185] C. Chopard, G. Bertho and T. Prange, *RSC Advances* **2012**, 2, 605-615.

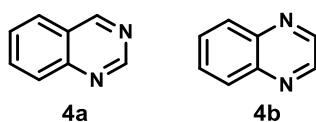
¹⁸⁶ [186] S. Yildirim, T. T. Franko, R. Wohlgenuth, H.-P. E. Kohler, B. Witholt and A. Schmid, *Advanced Synthesis & Catalysis* **2005**, 347, 1060-1072.

¹⁸⁷ [187] T. C. Fischer, B. Cerra, M. J. Fink, F. Rudroff, E. Horkel and M. D. Mihovilovic, *European Journal of Organic Chemistry* **2015**, 2015, 1464-1471.

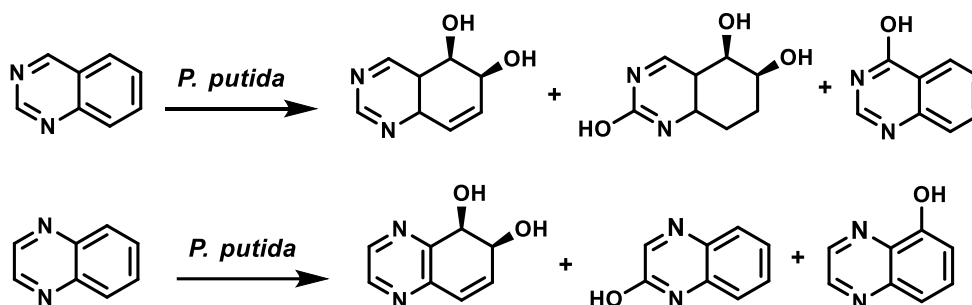
E. coli JM109(DE3)(pDTG141).¹⁸⁵ In 2012 Chopard et al. reported conversion of 1-methylisoquinoline and 3-methylisoquinoline to corresponding 7,8-dihydrodiols with moderate to full conversions. Monohydroxylation of methyl substituent was, however, major competing reaction and had twice the relative yield to that of the dihydrodiols.¹⁸⁵ Isoquinoline-1-carbonitrile (**3b**) was previously not reported as substrate. Like quinoline-2-carbonitrile all three regioisomers were expected to be formed.

Isoquinoline-3-carbonitrile (**3d**) was previously not reported substrate. Both products of transformation on the carbocyclic ring of isoquinoline-3-carbonitrile **3d** were expected to be formed.

2.12.4 Quinazoline and quinoxaline



Boyd reported the conversion of **4a** and **4b** with *P. putida* UV4 to corresponding hydroxylated products.^{170,171}



Scheme 2-22 Biotransformations of fused bicyclic bisazaarenes by the TDO from *P. putida* as reported by Boyd.¹⁷⁰

In 2012 Chopard reported biotransformation of fused bicyclic bisazaarenes by the NDO system from *Pseudomonas sp.* NCIB 9816-4. Quinoline was converted to corresponding 5,6-dihydrodiol as the only metabolite with NDO. When quinazoline was biotransformed with NDO, 5,6-dihydrodiol and quinazoline-8-ol was formed which was not previously reported.¹⁸⁵

3 Scope and perspective of the thesis

This thesis aimed at the investigation of biooxidations in general and use of dioxygenase biotransformations to synthesize dihydrodiols of PAHs and azaarenes (NPAHs), in details.

Azaarenes are widely distributed throughout environment and some members of this group exert highly toxic effects.¹² Quinoline is one of the most abundant and has been reported to have carcinogenic and mutagenic effects in nature.^{13,14} Several bacterial strains are able to metabolize some polycyclic azaarenes.¹⁷

3.1 Bioremediation of azaarenes

Bioremediation is one of the strategies applied to rehabilitate polluted environments.^{15,16} Due to the large number of enzymes being produced in native whole-cells the efficiency of biotransformations is limited. As a consequence, non-natural substrates can be converted to unwanted side-products due to competing reactions with a variety of enzymes. Similarly, the desired product can further converted by another competing enzyme to be introduced into metabolic pathways. To overcome these problems, recombinant expression systems offer the advantage to increase the production and availability of the required enzyme by suppressing or eliminating all other biocatalytic entities present in a living cell. Recombinant whole-cell biotransformations offer a great range of flexibility and adaptability in order to achieve excellence in biocatalytic synthesis.¹⁸⁸

3.2 Dioxygenases and azaarenes

Pseudomonas putida UV4, which expressing toluene dioxygenase (TDO),^{9,10} has been shown to transform monocyclic arenes to primary metabolites. It has been also reported to transform quinoline and other bicyclic azaarenes, but in low concentrations. Naphthalene dioxygenase (NDO) uses naphthalene as its natural substrate for conversion. As quinoline is almost isosteric to naphthalene, theoretically, a bacterial strain expressing naphthalene dioxygenase should be a better choice. is better choice for the conversion of NPAHs with

¹⁸⁸ [188] M. T. Reetz, *Angewandte Chemie* **2002**, *114*, 1391-1394.

higher yields.⁸ NDO-catalysed biotransformation of fused bicyclic azaarenes should be expected to occur in good yields, providing less toxic compounds as metabolites.

3.3 Substrate selection for dioxygenases

Earlier studies on azaarenes have shown the diols could be formed by *cis*-dihydroxylation at 5,6-bond and 7,8-bond of the carbocyclic ring. It is also shown to occur at 3,4-bond of heterocyclic ring resulting in rather unstable diols which decompose resulting in formation of mono-hydroxy products by the elimination of water. Unsubstituted quinolines and isoquinolines are reported to produce all three possible diols with *Pseudomonas putida* UV4 containing TDO (Boyd 2002).¹⁸⁴ There is vast diversity of substrate acceptance and relative ratio of preferred diol formation with the substituted azaarenes. It depends on number of factors including type of recombinant system, position, size, and polarity of substituent. There are also few examples in the literature representing monohydroxylation of the alkyl group regardless of its position on carbocyclic or heterocyclic ring. Monohydroxylation of alkyl substituent is kind of competing reaction with the diol formation as alkyl side chain hydroxylated metabolites generally stay in the aqueous phase without undergoing further dihydroxylation and forming triols. So far there are examples of *cis*-diol formation of monosubstituted azaarenes where as there are very few examples of diols formed by *cis*-dihydroxylation of polysubstituted azaarenes. This might be due to different electronic effect of substituents and active site of enzyme. Monocyclic aromatic compounds with two substituents are reported to be the substrates for dihydroxylation.

Earlier studies from collaborating groups^{189,190,191,192,193} revealed that cyano and ester substituted azines can serve as valuable starting compounds for subsequent transformations. Thus, investigation of such derivatives seemed also of practical importance. Because of the relatively easy availability, cyano and ester substituted isoquinoline and quinoline derivatives have been selected for the present study.

¹⁸⁹ [189] S. Batori, G. Hajos, P. Sandor and A. Messmer, *Journal of Organic Chemistry* **1989**, *54*, 3062-3068.

¹⁹⁰ [190] G. Hajos, Z. Riedl and A. Messmer, *Acta Chem. Scand.* **1993**, *47*, 296-301.

¹⁹¹ [191] M. Beres, G. Hajos, Z. Riedl, G. Timari and A. Messmer, *Monatsh. Chem.* **1998**, *129*, 897-908.

¹⁹² [192] M. Beres, G. Hajos, Z. Riedl, T. Soos, G. Timari and A. Messmer, *Journal of Organic Chemistry* **1999**, *64*, 5499-5503.

¹⁹³ [193] L. Filaok, T. A. Rokob, G. A. Vasko, O. Egyed, A. Gomory, Z. Riedl and G. Hajos, *Journal of Organic Chemistry* **2008**, *73*, 3900-3906.

3.4 Synthetic importance of azaarenes

Quinoline or isoquinoline moieties are present in many classes of biologically- active compounds. New metabolites could give access to modifications of the quinoline or isoquinoline moieties which can trigger interesting biological activities.

Within this contribution we have focused our attention to substituted quinolines and isoquinolines. While such systems have been investigated in the past, the chosen substituents (Cl, Br, OMe) adjacent to the ring nitrogen atom represented labile functionalities, which led to subsequent hydrolytic processes and ultimately to complicated compound mixtures. Based on rich chemistry associated to carboxylic acid derivatives and nitrile groups and the hydrolytic stability of the corresponding quinoline and isoquinoline derivatives, we investigate a small collection of such compounds in NDO mediated biooxygenations. The biocatalyst originated from *Pseudomonas putida* and was utilized within recombinant whole-cells of *Escherichia coli*.

Benzoate dioxygenase was used in order to see the formation of possible 1,2-dihydroxylation. As this enzyme class is mainly responsible of incorporating molecular H₂ on the carbocyclic or heterocyclic ring containing the functional groups. There are quite few examples in literature this enzyme class working on a number of monocyclic aromatic compounds. We wanted to try few bicyclic compounds of both carbocyclic and heterocyclic type with both electron donating and withdrawing functional groups in order to see the conversion and product formation. TDO is most studied enzyme as far as the dihydroxylation of monocyclic aromatic hydrocarbons is concerned. NDO was aimed to study under different conditions and different concentrations of PAHs and NPAHs.

4 Results and discussion

4.1 Biotransformations

A major deliverable of this work was to expand the substrate profile of available dioxygenase enzymes. Various bicyclic and polycyclic PAH and NPAH substrates were subjected to screening scale experiments with both 1,2-dioxygenase and 2,3 dioxygenase classes of enzymes. Successful transformations were repeated on preparative and large scale in order to establish the stereochemistry and absolute configuration of products formed by the reactions with dioxygenases. Substrates with nitrile substituent, amongst others, were of interest to generate new metabolites which would then lead to powerful synthons for further synthetic transformations.

4.2 Initial screening of substrates

Preparation of biological culture media used for biotransformations is given in details in the experimental section of this thesis. We have used growing cells approach to transform the substrates which has worked for us. Screenings of selected substrates were performed with dioxygenase from *Ralstonia eutropha* B9 expressing TDO, *P. putida* JM109 (DE3) (pDTG141) expressing NDO (NDO1), and *P. putida* JM109 (PVL 1343 + PMS13) also expressing NDO (NDO2). Complete screening methods are discussed in the experimental section of the thesis. The metabolites were analyzed by HPLC (method described in section 5.4.1) and GC (method described in section 5.4.2) after biotransformations with this recombinant whole cell system.

4.2.1 Screening with *Ralstonia eutropha* B9 expressing BZDO

As described in section 2.8 dioxygenase expressed in *Ralstonia eutropha* B9 belongs to 1,2-dioxygenases family and is mainly responsible for dihydroxylation of benzoates. Substrates tested were quinoline-2-carbonitrile (**2a**), isoquinoline-1-carbonitrile (**3b**) and isoquinoline-3-carbonitrile (**3d**). Screening scale experiments were carried out according to method GP1 described in experimental section 5.5.1. It revealed only the starting materials by HPLC analysis and thus no conversion could be detected. After the screening with these

substrates did not show any successful transformations, no further substrates were tested with *Ralstonia eutropha* B9.

4.2.2 Screening with *E. coli* JM109 (pDTG601A) expressing TDO

Screening scale biotransformations were performed with *E. coli* JM109 (pDTG601A) expressing TDO according to GP2. Substrates screened with TDO were accepted in low to moderate concentrations by the enzyme. In almost all the cases substrates converted by TDO were also converted by NDO1 and NDO2. HPLC analysis of the metabolites suggested generally higher substrate conversions and product formation for NDO1 and NDO2 as compared to TDO. Therefore, preparative scale biotransformations were carried out with the enzyme which gave best screening scale results. Screening scale results, however, are summarized in corresponding tables for individual enzymes.

4.2.3 Screening with JM109 (DE3) (pDTG141) expressing NDO (NDO1)

Screening with JM109 (DE3)(pDTG141) expressing NDO was carried out in MSB medium by using same method described for TDO in the previous section.

4.2.4 Screening with JM109 (PVL 1343 + PMS13) expressing NDO (NDO2)

Screenings with recombinant *E. coli* JM109 (pVL1343pMS13) expressing naphthalene dioxygenase was carried out in MSB with same general procedure as given for TDO in section 3.2.3. The difference being the amount of antibiotics supplemented with ampicillin 100 $\mu\text{g mL}^{-1}$ and kanamycin 50 $\mu\text{g mL}^{-1}$ and induction by salicylic acid (as dioxane solution, 2 mM L^{-1} of MSB).

Among all the dioxygenases studied NDO2 has shown very promising and outstanding results towards the dihydroxylation of PAHs and azaarenes. It could be due to the dioxygenase broad substrate acceptability and also due to the induction with salicylic acid. In the literature there is relative evidence of salicylic acid induction giving two to three fold increased yields as compared to induction with IPTG.

4.3 Results of screening scale experiments

1-Methylnaphthalene (1b)

1-Methylnaphthalene (**1b**) was converted by all three dioxygenases and a mixture of two products was formed. TDO showed 25% conversion, NDO1 showed 21% conversion and NDO2 showed 13% conversion.

2-Methylnaphthalene (1c)

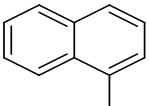
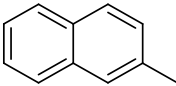
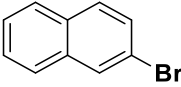
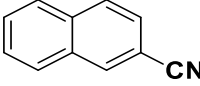
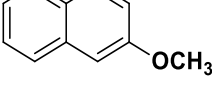
2-Methylnaphthalene (**1c**) was converted by all three dioxygenases. TDO showed 20% conversion, NDO1 showed 32% conversion and NDO2 showed 62% conversion and one product was observed in HPLC.

2-Bromonaphthalene (1d)

Screening with TDO resulted in full conversion after 48 hrs. Screening with NDO1 and NDO2 also gave full conversion after 48 hrs and one product peak was observed.

All the screening results naphthalene type substrates are summarized in the table 4-1.

Table 4-1 Results of screening scales experiments with naphthalenes

Entry	No.	Substrate	TDO		NDO1		NDO2	
			conv	ratio	conv	ratio	conv	ratio
1	1b		25	60, 40	20	70, 30	15	45, 55
2	1c		20	100	32	100	62	100
3	1d		> 95	100	> 95	100	> 95	100
4	1e		40	100	45	100	82	100
5	1f		10	75, 25	15	70, 30	18	72, 28

Naphthalene-2-carbonitrile (1e)

Naphthalene-2-carbonitrile (**1e**) was converted 40% by TDO, 45% by NDO1, and 82% by NDO2 showing single product peak.

2-Methoxynaphthalene (1f)

Screening with 2-methoxynaphthalene (**1f**) showed formation of 2 products with all three dioxygenases and conversion in the range of 30- 50% after 48 hrs.

Quinoline-2-carbonitrile (2a)

Quinoline-2-carbonitrile (**2a**) was not fully converted by either of dioxygenase. After 48 hrs TDO showed 10%, NDO1 showed 38%, and NDO2 showed 90% conversion respectively. Screening with BZDO did not show any conversion and only starting material peaks were observed by HPLC.

Methyl quinoline-2-carboxylate (2b)

Methyl quinoline-2-carboxylate (**2b**) was not converted by any of dioxygenases. It had solubility problem in the media and was recovered unconverted after 48 hrs. Screening with BZDO did not show any conversion and only starting material peaks were observed by HPLC.

Quinoline-2-carboxylic acid (2c)

Substrate acceptance was very little with NDO1 and TDO and only traces of diol were observed. Reaction with NDO2 showed the up to 10% formation single product.

Quinoline-2-carboxamide (2d)

Quinoline-2-carboxamide (**2d**) had about 10% conversion with TDO after 48 hrs. NDO1 had less than 15% conversion after 48 hrs. NDO2 had 20% conversion after 48 hrs. Screening with BZDO did not show any conversion and only starting material peaks were observed by HPLC.

2-(Tribromomethyl)-quinoline (2e)

Screening of 2-(tribromomethyl)-quinoline (**2e**) with NDO1 showed 22% conversion after 24 hrs and 33% conversion after 48 hrs. TDO showed 50% conversion after 48 hrs whereas NDO2 showed full conversion after 24 hrs.

Bisquinoline (2f)

Bisquinoline (**2f**) had solubility problems in the MSB. Bisquinoline was added as solution in dioxane but precipitated as soon as added to the medium. It remained precipitated in the medium and with 48 hrs of shaking stayed unconverted. β -Cyclodextrin was added in the medium in order to facilitate the solubility of bisquinoline but it had no effect on solubility and no conversion was observed. Starting material remained unconverted in the medium and could be extracted without any consumption or decomposition.

3-Methylquinoline (2g)

3-Methylquinoline (**2g**) showed no conversion with TDO and NDO2. HPLC analysis of aliquots showed only starting material peaks. In one experiment with NDO1 40% conversion after 48 hrs.

3-Bromoquinoline (2h)

Screening of 3-bromoquinoline (**2h**) with TDO showed conversion of less than 10% after 24 hrs. NDO1 showed 30% conversion after 24 hrs and upto 70% after 48 hrs. NDO2 showed 48% conversion after 24 hrs and full conversion after 48 hrs. NDO1 and TDO showed single products whereas NDO2 showed formation of 2nd minor product.

3-Bromoquinoline-2-carboxamide (2i)

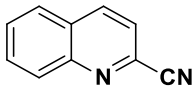
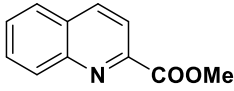
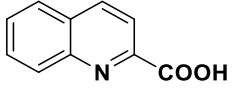
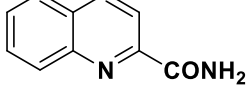
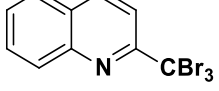
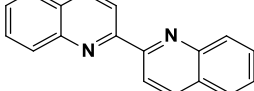
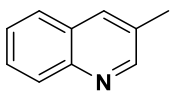
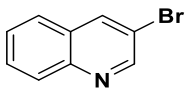
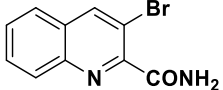
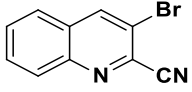
3-Bromoquinoline-2-carboxamide (**2i**) had solubility problems. It was added as solution in dioxane but precipitated as soon as added to the medium. It remained precipitated in the medium and with 48 hrs of shaking stayed unconverted. β -Cyclodextrin was added in the medium in order to facilitate the solubility but it had no effect on solubility and no conversion was observed. Starting material remained unconverted in the medium and could be extracted without any consumption or decomposition.

3-Bromoquinoline-2-carbonitrile (2j)

3-Bromoquinoline-2-carbonitrile (**2j**) had solubility problems. It was added as solution in dioxane but precipitated as soon as added to the medium. It remained precipitated in the medium and with 48 hrs of shaking stayed unconverted. β -Cyclodextrin was added in the medium in order to facilitate the solubility but it had no effect on solubility and no conversion was observed. Starting material remained unconverted in the medium and could be extracted without any consumption or decomposition.

All the screening results are summarized in the table 4.2.

Table 4-2 Table 4-2: Results of screening scales experiments with quinolines

Entry	No.	Substrate	TDO		NDO1		NDO2	
			conv	ratio	conv	ratio	conv	ratio
1	2a		10		38		90	
2	2b		n.c.		n.c.		n.c.	
3	2c		5		5		10	
4	2d		10		15		20	
5	2e		n.c.		n.c.		n.c.	
6	2f		n.c.		n.c.		n.c.	
7	2g		2		2		2	
8	2h		5-10		48		70	15:1
9	2i		n.c.		n.c.		n.c.	
10	2j		n.c.		n.c.		n.c.	

1-Methylisoquinoline (3a)

1-Methylisoquinoline (**3a**) was converted less than 5% after 48 hrs by TDO and NDO2. NDO1 showed 5% conversion after 24 hrs and 10% conversion after 48 hrs.

Isoquinoline-1-carbonitrile (3b)

Screening scale biotransformation of Isoquinoline-1-carbonitrile (**3b**) with TDO resulted in less than 5% conversion and traces of two products. NDO2 had relatively better

acceptance and about 15% substrate was converted showing two peaks in HPLC with relative ratio of 65:35. Screening with NDO1 showed 22% conversion after 48 hrs and 60:40 ratio of two products formed. Screening with BZDO did not show any conversion and only starting material peaks were observed by HPLC.

3-Methylisoquinoline (3c)

3-Methylisoquinoline (**3c**) was partially converted by all dioxygenases and none of them reached full conversion after 48 hrs. Screening with TDO showed traces of product after 24 hrs and 32% conversion after 48 hrs. Screening with NDO1 showed 10% conversion after 24 hrs and 36% conversion after 48 hrs while with NDO2 showed 20% conversion after 24 hrs and 25% conversion after 48 hrs.

Isoquinoline-3-carbonitrile (3d)

Screening scale biotransformation of isoquinoline-3-carbonitrile (**3d**) with TDO showed up to 20% conversion after 48 hrs giving predominantly one major product and traces of second product. Conversion with NDO1 was 25% showing two peaks in HPLC with relative ratio of 90:10. Screening with NDO2 showed 52% conversion after 48 hrs and 85:15 ratios of two products formed. Screening with BZDO did not show any conversion and only starting material peaks were observed by HPLC.

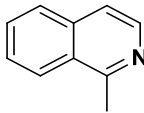
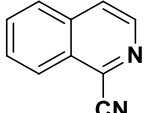
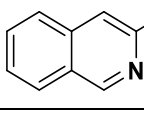
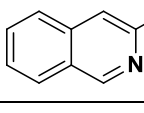
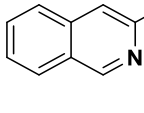
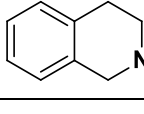
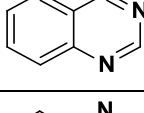
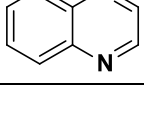
Methyl isoquinoline-3-carboxylate (3e)

Methyl isoquinoline-3-carboxylate (**3e**) had solubility problems. It was added as solution in dioxane but precipitated as soon as added to the medium. It remained precipitated in the medium and with 48 hrs of shaking stayed unconverted. β -Cyclodextrin was added in the medium in order to facilitate the solubility but it had no effect on solubility and no conversion was observed. Starting material remained unconverted in the medium and could be extracted without any consumption or decomposition. Screening with BZDO did not show any conversion and only starting material peaks were observed by HPLC.

1,2,3,4-Tetrahydroisoquinoline (3f)

No product peaks were observed in HPLC with NDO1 and NDO2. Also starting material peak was not observed after 24 hrs of biotransformation. Screening with TDO showed traces of product peak as calculated by HPLC. All the screening results are summarized in the table 4.3.

Table 4-3 Results of screening scales experiments with Isoquinolines

Entry	No.	Substrate	TDO		NDO1		NDO2	
			conv	ratio	conv	ratio	conv	ratio
1	3a		2		5		2	
2	3b		5		22	60:40	15	65:35
3	3c		32		36		25	
4	3d		10	90:10	25	90:10	52	85:15
5	3e		-		-		-	
6	3f		n.c		n.c		n.c	
7	3g		5		30	60:40	40	70:20:10
8	3h		5		5		11	

Quinazoline (3g)

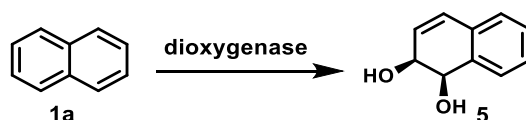
Quinazoline (**3g**) was converted partially about 30% after 48 hrs with NDO1. There were three product peaks with relative ratio of 60%, 40% and 10%. Screening with NDO2 had about 40% conversion and showed three products in relative ratio of 70%, 20% and 10%. TDO had upto 5% conversion after 48 hrs.

Quinoxaline (3h)

Quinoxaline (**3h**) had traces of product with TDO and NDO1 (4-5%) after 24 hrs and conversion was not increased after 48 hrs. NDO2 had 11% conversion after 24 hrs and 16 % after 48 hrs. TDO showed less substrate acceptance and NDO2 showed maximum substrate conversion.

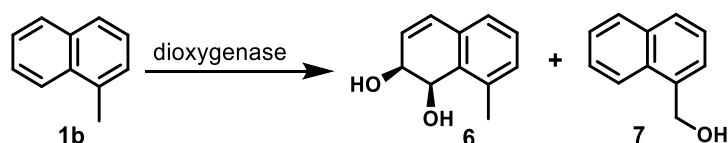
4.4 Preparative scale biotransformations

4.4.1 Naphthalene (1a)



A 100 mg scale transformation with NDO2 in the shake flask experiment resulted in full conversion after 24 hrs. Purification of diol The by using MPLC and PE:EtOAc (4:1 to 1:1) gave 88% pure *cis*-1,2-dihydro naphthalene-1,2-diol **5**. Biotransformation was performed with NDO2 in 2 L MSB distributed in 1L and 2L shake flasks and supplied with necessary antibiotics. Dioxygenase genes were induced for 3 hrs by addition of 2 mM salicylic acid when the culture turbidity at 600 nm reached approximately 1.5. Cells were collected by centrifugation, washed twice and resuspended in MSB, and naphthalene was added concentration of 2 g L⁻¹ naphthalene as solution in dioxane. The level of glucose was restored when needed. After completion of reaction, fractions were combined, cells were centrifuged off and supernatant was concentrated under vacuum.

4.4.2 1-Methylnaphthalene (1b)



Screening scale biotransformation with showed two products in the HPLC. The substrate conversion with TDO was observed as 25% (55, 42), NDO1 21% (65, 29), and NDO2 40% (40, 52).

Biotransformation of 1-methylnaphthalene with NDO1 was carried out in 2L MSB media and at 1g L⁻¹ scale. It showed the similar trend with TDO and NDO2 and same products were formed. Loss of starting material was observed in this matter as even though there was less than 30% conversion; starting material recovery was less than half. Extraction was done by base treated EtOAc and crude material after drying was subjected to MPLC. Three fractions were isolated along with 15% unreacted starting material. Monohydroxylation occurred on CH₃ group and compound **7** was produced in 5% yield. There was another

uncharacterized minor fraction about 40 mg which does not seem to be monooxygenated or dioxygenated product. Dihydroxylated product being 7,8-diol **6** was 10% formed.

Biotransformation of 1-methylnaphthalene with TDO was carried out in 1L MSB media and at 1g L⁻¹ scale. It showed the similar trend as that of NDO1 and NDO2 and same products were formed. Substrate acceptance was comparable to NDO1 and crude yield was about 70%. Purification of products was done by MPLC of crude material. Three fractions were isolated along with traces of starting material. Monohydroxylation occurred on CH₃ group and compound 10 was produced in 8% yield. There was another uncharacterized minor fraction about 40mg which does not seem to be mono or deoxygenated product. Dihydroxylated product being 7,8-diol was 14% formed.

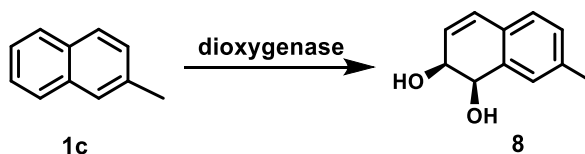
Biotransformation of 1-methylnaphthalene in 3L of media was carried out as described in experimental section in details. Loss of starting material was also observed as crude yield was roughly 70%. About 500 mg of starting material was recovered mainly due to loss. NDO2 has substrate acceptance better than both NDO1 and TDO producing 20% diol from 3g of starting material. The products formed by all three systems were similar although the substrate acceptance and product ratios were slightly different. Both spectral and physical properties are in accordance with the literature.

No dihydroxylation on 5,6-position was observed on the substrate by either of the dioxygenases however, careful analysis of crude NMR samples showed the traces of 5,6-diols. Relative yields are summarised in the table given below.

Table 4-4 Dihydroxylation of 1-Methylnaphthalene

	TDO	NDO1	NDO2	Ref 182	Ref. 183
Substrate	1.0 g	2.0 g	3.0 g	0.5 g	1.0 g
MSB Medium	1L	2L	3L	1L	1L
Conversion %	25	21	40		
1-methyl alcohol	8% (90 mg)	5 % (100 mg)	13 % (427 mg)		
5-6 diol	not detected	not detected	not detected		
7-8 diol	14 % (168 mg)	10% (230 mg)	20 % (732 mg)	5.9 %	158 mg L ⁻¹

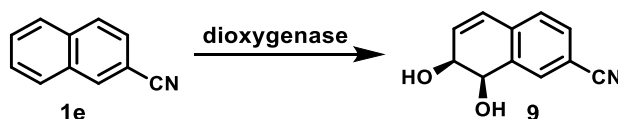
4.4.3 2-Methylnaphthalene (1c)



Screening scale biotransformation of 2-methylnaphthalene **1c** was studied with three dioxygenases. Three different reactions pairs were set up with three enzymes respectively. Product formation was monitored by using HPLC analysis and samples taken every 12 Hrs. TDO showed only traces of product after 24 hrs and there was less than 20% relative product formation after 72 Hrs. NDO1 showed product formation in relatively larger proportion with almost no further product formation after 36 hrs. Maximum product formation as analyzed by HPLC studies was less than 30%. NDO2 started slow conversion of 2-methylnaphthalene and after 48 hrs reached till 60%.

Preparative 200 mL scale biotransformation of 2-methylnaphthalene was carried out only with NDO2 in 1L MSB. After 48 hrs reaction was stopped and worked up. The crude dried product mixture was purified with MPLC giving 7,8-diol **8** as the only product. The dihydrodiol formed was *cis*-(1R,2S)-7-methyl-1,2-dihydronaphthalene-1,2-diol **8**. It is mentioned here as 7,8-diol only with reference to the CH₃- group of the starting 2-methylnaphthalene in order to understand the regiochemistry of dihydroxylation. Traces of 2nd minor diol were also observed in the NMR with relative percentage of less than 5% but could not be isolated at small scale experiments. Most probably it is corresponding 5,6 diol of 2-methylnaphthalene as peaks compare to the literature.¹⁸³ 7,8-diol formation is in accordance with the results previously reported by Hudlicky¹⁷⁹ and Boyd¹⁸³ whereas corresponding 3,4-diol was not observed.

4.4.4 Naphthalene-2-carbonitrile (1e)



In 2008 Kwitt et al¹⁸³ reported biotransformation of 6 substituted naphthalenes (H, Br, F, OMe, CN, Me, I) at 2-position using TDO. Total of nineteen dihydroxylation products were reported with their chemical and physical properties. In order to reproduce some of the literature products and learn the behavior of dioxygenases, 2-methyl naphthalene **1c** and naphthalene-2-carbonitrile **1e** were selected. *cis*-(1R,2S)-7-methyl-1,2-dihydronaphthalene-

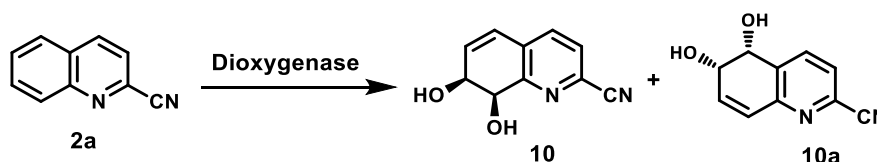
1,2-diol (**8**) obtained from biotransformation of 2-methylnaphthalene **1c** was in accordance with the literature as mentioned in section 4.4.3 and 5.6.4. *cis*-(5R,6S)-3-bromo-5,6-dihydroquinoline-5,6-diol (**9**) differed significantly from the literature whereas it was in complete accordance with our own laboratory results. To elaborate further we performed the experiments twice and always obtained similar results. Although the absolute configuration and conformation was according to the literature, NMR spectra and physical properties were different. We contacted via email to the corresponding authors in order to clarify and correct the literature. The authors responded that the main emphasize of the paper was on assignment of absolute configuration and conformations from Circular Dichroism (CD) and Optical Rotation Measurements (OR). That is obvious from the paper as CD and OR studies of dihydroxylation products of dioxygenases can help to assign the absolute configurations.

While according to the literature all three possible products of dihydroxylation (7-8 diol = 15%, 5,6-diol = 57%, 3,4-diol = 28%) were formed by using *Pseudomonas putida* expressing TDO enzyme.

Biotransformation of naphthalene-2-carbonitrile **1e** (2g scale) was carried out in 3L MSB medium by using shake flask experiments. Reaction was completed in 36 hrs and worked up according to general procedure. Product was purified by MPLC (silica gel, gradient chromatography, EtOAc: hexane, 30% EtOAc 20 min, 50% EtOAc 20 min, 50%-100% EtOAc 20 min, 100% EtOAc 20 min). *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydronaphthalene-2-carbonitrile (**9**) was only major isolated product of reaction and was produced in 86% yield. Previously, it was not isolated as product of biotransformation rather synthesized chemically by the exchange of I from 7,8-diol of 2-iodonaphthalene. Traces of 5,6 diol were also observed in the crude NMR with TDO.

4.4.5 Quinoline-2-carbonitrile (**2a**)

Quinoline-2-carbonitrile (**2a**) was studied extensively with dioxygenases under different conditions. In order to optimize the substrate acceptance, growth medium, temperature, and OD it was subjected to biotransformation with *Ralstonia eutropha* B9, TDO, NDO1 and NDO2. The optimized conditions were then used for biotransformation of other quinoline and isoquinoline substrates with small modifications when required.



Biotransformation with *Ralstonia eutropha* B9 was not successful both with and without induction using method and conditions mentioned in details in section 5.5.1. There was no transformation observed and starting material was recovered after 48 hrs of screening experiments.

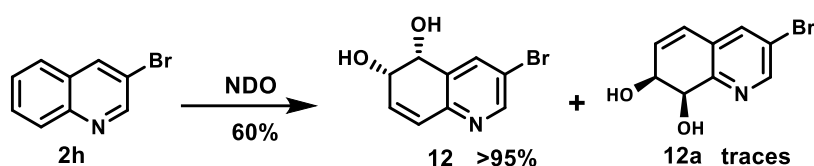
It's also important to mention that although induction of naphthalene dioxygenase (IPTG in case of NDO1 and TDO and salicylic acid in case of NDO2) gave higher conversions, lower conversions were also obtained when substrate was added without induction. Biotransformations without induction resulted in formation of same diols but generally in lower amounts. Concerning the regioselectivity of the transformation, dihydroxylated products **10** and **10a** could be formed with other possibility of 3,4-diol that was not observed. However, diol **10** was obtained as the exclusive product of oxygenation whereas diol **10a** was observed in trace amounts by careful examination of spectral data of isolated fractions.

Screening scale biotransformation with NDO1 and TDO in Mineral M9 medium was not successful as expected but the traces of dihydroxylated products were observed by HPLC analysis. The conversion was increased when culture medium was changed from Mineral M9 to MSB. Similar results were obtained when tried with NDO2 in Mineral M9 and MSB. Mineral M9 medium was abandoned in favor of MSB. Only single dihydroxylated product was observed with all three dioxygenases.

Substrate acceptance studies under shake flask experiments showed that TDO had least conversion of **2a** with maximum 20 mg 100 mL⁻¹. When higher concentrations of **2a** were added the conversion dropped to maximum of 25 mg 100 mL⁻¹ indicating higher substrate concentrations had substantial effect on the performance of growing cells. Substrate conversion with NDO1 was 60mg 100 mL⁻¹ whereas NDO2 conversion was in range of 75mg 100 mL⁻¹ of MSB. Preparative scale biotransformations were generally carried out 1L and 2L baffled shake flasks containing 20% v/v of medium giving comparable yields to the fermenter. It was also subjected to transformation in 2L fermenter details discussed in fermentation section. The successful conversions in 2L fermenter were 1g with NDO1 with 82% isolated yield and 2g with NDO2 with 72% isolated yield.

4.4.6 3-Bromoquinoline (2h)

TDO showed conversion of less than 10% after 24 hrs. NDO1 showed 30% conversion after 24 hrs and up to 70% after 48 hrs. NDO2 showed 48% conversion after 24 hours and full conversion after 48 hrs. NDO1 and TDO showed single products as described according to the literature whereas NDO2 showed formation of 2nd minor product also which could be result of dihydroxylation at 7,8-position. In order to isolate the minor diol **12a**, biotransformation was carried out with NDO2. Minor diol **12a** could not be separated from the major diol **12** after several attempts. The NMR of crude mixture follows the pattern of 5,6- and 7,8- diols as compared with results from other compounds.



Preparative scale biotransformation was performed with both NDO1 and NDO2. Pale yellow crystalline material was isolated being *cis*-(1R,2S)-7-methyl-1,2-dihydronaphthalene-1,2-diol (**14**). Fig. 4-1 shows ¹H NMR of pure 5,6-diol **12**. Fig. 4-2 shows ¹H NMR of mixture of 5,6- and proposed 7,8- diols. ¹H NMR signals at δ 4.46 ppm (dd, $J = 6.2, 4.0$ Hz, 1H) and 4.70 ppm (d, $J = 4.9$ Hz, 1H) represent H7 and H8. ¹H NMR signals at 6.33 ppm (dd, $J = 9.5, 5.6$ Hz, 1H) and 6.58 ppm (d, $J = 9.7$ Hz, 1H) correspond to olefinic H5 and H6. The aromatic region might have ¹H signals between 7.4 ppm and 7.7 ppm ppm from aromatic protons of starting material and ¹H signals at 8.30 ppm (d, $J = 3.9$ Hz, 1H), and 8.50 ppm (d, $J = 2.1$ Hz, 1H) correspond to heterocyclic ring of *cis*-(7S,8R)-3-Bromo-7,8-dihydroquinoline-7,8-diol **12a**.

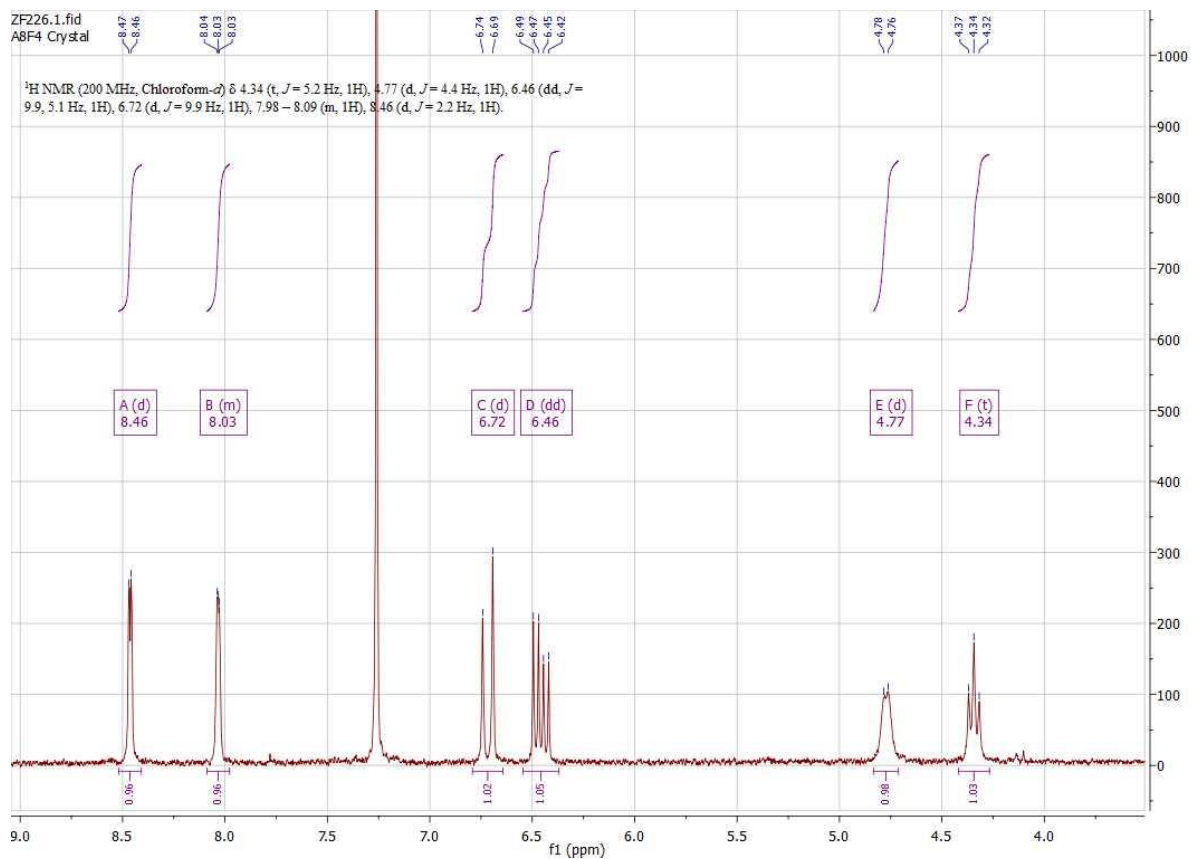


Fig. 4-1 ¹H NMR of pure 5,6-diol

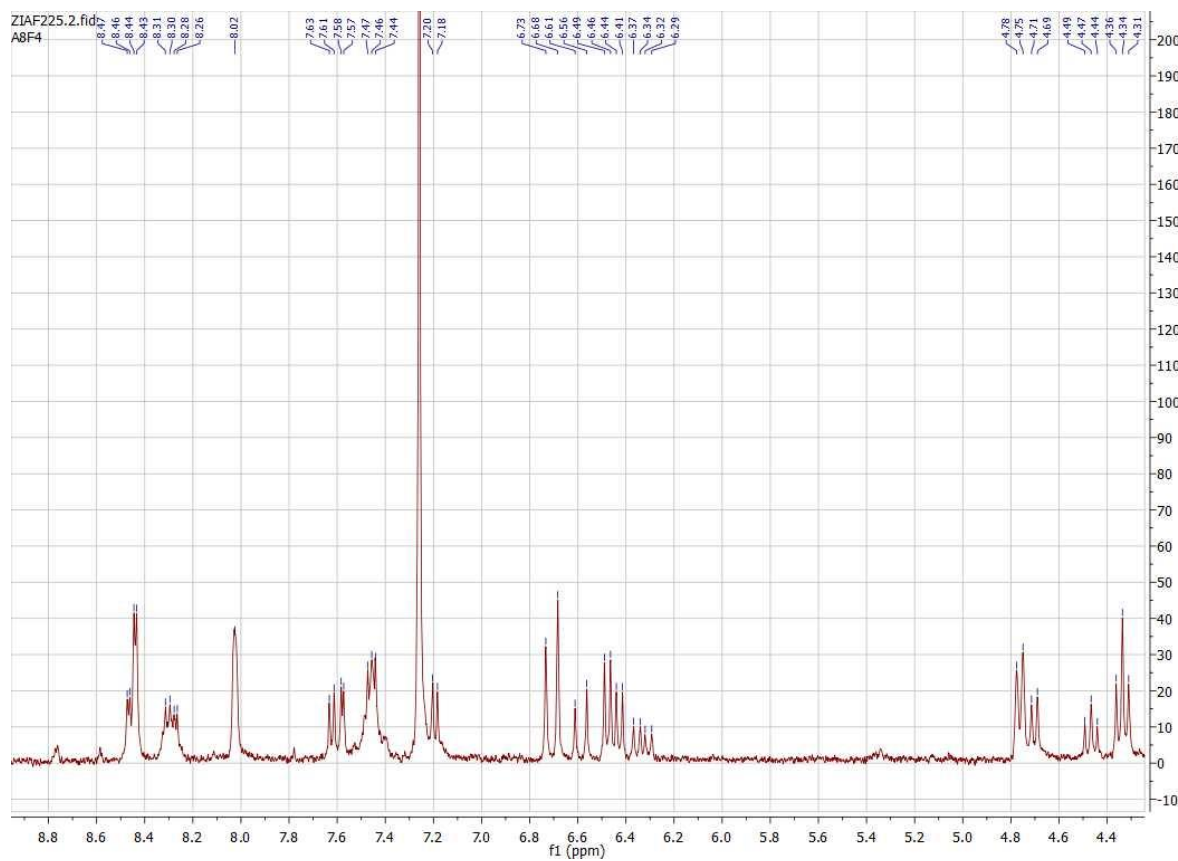
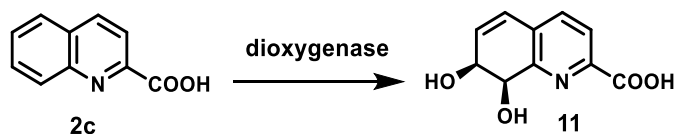


Fig. 4-2 ¹H NMR of mixture of 5,6- and proposed 7,8- diol

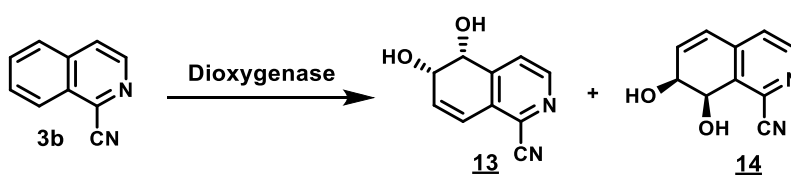
4.4.7 Quinoline-2-carboxylic acid (2c)



In an attempt to test quinoline-2-carboxylic acid as substrate screening scale reactions were performed using NDO1, TDO and NDO2. Analysis of samples from biotransformation media showed the formation of single dihydroxylation product. Substrate acceptance was very little with NDO1 and TDO and only traces of diol were observed. Reaction with NDO2 showed the up to 10% formation of diol. Larger scale shake flask experiments were set up in order to see the regioselectivity of transformation. Although dihydroxy diol was produced, it was extremely difficult to isolate it from the parent acid. Various unsuccessful attempts were made in order to recover the diol in pure form for analytical purpose. My research colleague had studied multi gram scale dihydroxylation of benzoic acid with *Ralstonia eutropha* B9 and precipitating the resulting diol as sodium salt.¹³⁴

This method was not successful in this case mainly due to very low concentration of diol formed and in connection with the physicochemical properties of these compounds representing ionizable functions of carboxylic acids and amines. The crude NMR analyses of resulting diols are reported in the experimental section of this thesis.

4.4.8 Isoquinoline-1-carbonitrile (3b)



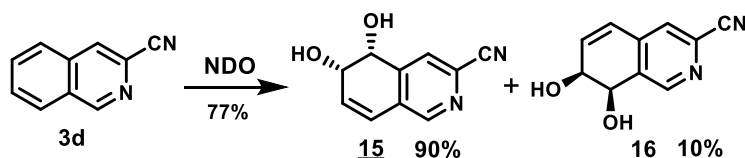
Isoquinoline-1-carbonitrile (**3b**) was previously not reported substrate. Theoretically, all three regioisomers could be produced but two products of dihydroxylation on carbocyclic ring were expected to be formed. It is very unlikely formation of unstable diol on the heterocyclic ring due to the presence of N and strong withdrawing CN group. Screening scale biotransformation with the recombinant strain expressing TDO had only traces of diol formation and overall less than 10% substrate was converted. Biotransformation with NDO2 showed similar behaviour as TDO and conversion was in the range of 10-15%. Increasing the media volume two fold and decreasing the substrate concentration had no major effect on the substrate acceptance of transformation. It also showed the limitation of NDO2 as poor

expression system for the transformation of 1-substituted quinoline. Screening scale transformation of isoquinoline-1-carbonitrile with JM109 (DE3)(pDTG141) containing naphthalene dioxygenase (NDO1) showed about 35% conversion and formation of two products in ratio of 4:3.

If compared with the results obtained by dihydroxylation of 1-methylnaphthalene **1b** with dioxygenases, similar trend of lower substrate acceptability is observed. However, in that case NDO2 performed better with 40% transformation as compared to the NDO1 and TDO with 21% and 25% conversion respectively. It can be concluded that the presence of N atom has some effect on the transformation and substrate acceptance for NDO2. It can be further elaborated by testing naphthalene-1-carbonitrile.

Preparative scale biotransformation of isoquinoline-1-carbonitrile **3b** was performed with NDO1 and using lower concentrations of substrate (200 mg L⁻¹ MSB). It was not completely transformed after 48 hrs and HPLC showed no further increase in diol peaks, therefore, it was worked up according to general procedure GP4. Mixture of two regioisomers was separated by MPLC using petrol ether and EtOAc as solvent mixture (silica gel 45g column, gradient chromatography, EtOAc: hexane, 30% EtOAc 10 min, 30-50% EtOAc 20 min, 50%-100% EtOAc 20 min, 100% EtOAc 20 min).

4.4.9 Isoquinoline-3-carbonitrile (**3d**)



Substrate **3d** was accepted by all three strains. Screening scale biotransformation with TDO showed about 10% conversion. Screening with NDO1 and NDO2 showed 25% and 52% conversion respectively. HPLC analysis showed the formation of two products in the ratio of ranging from 9:1 (TDO and NDO1) to 8:2 (NDO2). HPLC analysis of peaks and GC analysis of aliquots of **3d** showed the identical products formation by all NDO1, NDO2, and TDO, therefore, preparative scale biotransformations were carried out with better performing enzymes.

Preparative scale biotransformation of isoquinoline-3-carbonitrile **3d** was performed with NDO1 and using lower concentrations of substrate (300 mg L⁻¹ MSB). Substrate

acceptance with NDO2 was 700mg L^{-1} MSB. An excess of substrate would not only remain unconverted but also result in lower diol formation by recombinant strains. It was not completely transformed after 48 hrs and HPLC showed no further increase in diol peaks, therefore, it was worked up according to general procedure GP4. Mixture of two regioisomers was not separable by MPLC as using different solvent systems resulted in decomposition of minor diol. *cis*-(5R,6S)-5,6-dihydroxy-5,6-dihydroisoquinoline-3-carbonitrile **15** (85-90 %) was the major product whereas *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-3-carbonitrile **16** (10-15 %) was minor product of biotransformation.

As mixture of *cis*-diols **15** and **16** was not separated by column chromatography, efforts were made to get major diol to maximum purity. It was found that minor diol **16** having little more solubility could be removed by dissolving the mixture in ice-cold EtOAc. However, evaporation of cold EtOAc extracts generally resulted in yellowish brown gummy material as a result of decomposition. Major diol **15** was more stable as compared to the minor diol **16**.

Screening scale results showed higher relative ratio of minor diol **16** at up to 15% with NDO2 and NDO2 also expressed more than double the substrate acceptance as compared to NDO1. Preparative scale experiments with **3d** were done according to GP4 in case of NDO1 and GP5 in case of NDO2 whereas larger scale shake flask experiments were carried out according to GP6 and GP7 with NDO1 and NDO2 respectively.

4.5 Large Scale Biotransformations

Large scale biotransformations were performed with selected substrates. There were two methods tried in order to test the substrate acceptance and conversion on larger scale. First method used was to set up multiple shake flask experiments under the same set of conditions used for preparative scale. The substrate was evenly distributed in the flasks as dioxane solution and biotransformation was carried out. After completion of the reaction, the cells were centrifuged off and supernatant was combined and extracted for product isolation and purification. This method allowed using up to 3L media for biotransformation and substrate amounts of substrate in single batch with decent isolated yields. Larger scale biotransformations were also carried out in New Brunswick Bioflow 110 Fermenter equipment having 2L capacity.

4.5.1 Fermentation of naphthalene (1a)

Biotransformation of naphthalene was carried out multiple times with dioxygenases mainly to optimize the conditions. Naphthalene can be added to the medium also as solid without dissolving in dioxane and gets converted to corresponding dihydrodiol. As NDO1 acts under IPTG induction and NDO2 works under salicylate induction, it seems like later facilitates the dihydroxylation of naphthalene to higher extent.²¹¹ Table below gives an overview of different amounts of naphthalene converted after 36 hours of biotransformation with NDO1 and NDO2 using multiple shake flask experiments approach.

Table 4-5 Shake flask batch experiment with naphthalene

	NDO 1	NDO 2	NDO2
Substrate Amount	1 g L ⁻¹	2 g L ⁻¹	3 g L ⁻¹
Isolated Yield	80%	75%	67%

Biotransformation in fermenter was carried out at 30° C. Detailed procedure of fermentation with NDO1 and NDO2 is described in section 5.5.8 and 5.5.9 respectively. With the better control of oxygen flow and optimal fermentation conditions, larger substrate acceptance and improved yields were observed. As biotransformation with growing cells needs the cells to be harvested after reaching optimum OD, it was necessary to sterilize the whole fermentation system twice during the course of reaction.

Conditions used for biotransformation in fermenter are as follows,

Temperature: 30° C

Agitation: 300 rpm

Air Flow: 3 L min⁻¹

Carbon Source: 20 % glucose solution

Table 4-6 Fermentation conditions with NDO1 and NDO2

Dioxygenase	NDO1	NDO2
20% glucose	10 mL L ⁻¹	20 mL L ⁻¹
thiamine	300 mg L ⁻¹	30 mg L ⁻¹
Ampicillin	3 mL L ⁻¹	4 mL L ⁻¹
Kanaycin	n/a	2 mL L ⁻¹
Induction	2 mL of IPTG	2 mM salicylic acid

Naphthalene was added to the fermenter as 1,4-dioxane solution. Reaction was complete after 36 hours. Workup after the completion of reaction was as described in GP9. In order to avoid the consumption of large amount of solvent for product extraction it was from the aqueous media, it was concentrated under reduced pressure at 40°C to about 500 mL. This generally resulted in reduced solubility of diol in the nutrient rich aqueous media and efficiency of extraction was increased by stirring it with equal volume of EtOAc (500 mL) thrice. Biotransformation of 4 g of naphthalene in fermenter with NDO2 gave 80% isolated yield of diol **5**.

4.5.2 Fermentation of 1-methylnaphthalene (**1b**)

Biotransformation of 1-methylnaphthalene resulted in two products with all dioxygenases. Large scale biotransformation with **1b** was carried out using the combination of shake flask experiments as described in GP6 and GP7. The results are explained in section 4.4.2 summarized in table 4-4.

4.5.3 Fermentation of naphthalene-2-carbonitrile (1e)

Large scale fermentation was performed using benchtop fermenter. Single batch run was performed in order to perform biotransformation with growing cells under identical set conditions. Amount of substrate was selected on the basis of substrate acceptance observed during preparative scale shake flask experiments.

Table 4-7 Naphthalene-2-carbonitrile with different dioxygenases

	NDO 1	NDO 2	TDO
Substrate Added	1.0 g	3.0 g	0.70 g
Isolated Yield	75%	86 %	78%
5-6 diol	traces	traces	traces
7-8 diol	>95%	>95%	>95%

4.5.4 Fermentation of quinoline-2-carbonitrile (2a)

Large scale fermentation was performed using benchtop fermenter according to GP8 (with NDO1) and GP9 (with NDO2) respectively. There was no significant increase of product formation after 36 hours and the reaction was not completed after 48 hours. It was worked up and products were purified by MPLC. Results from large scale fermentations are summarized in table 4-7. Overall isolated yield with NDO1 was 65% and NDO2 was 71. Calculated yields on the basis of recovered starting material were 77% with both NDO1 and NDO2.

Table 4-8 Quinoline-2-carbonitrile with NDO1 and NDO2 in 2L Fermenter

	NDO 1	NDO 2
Substrate Added	500mg/1.8 L	2g/1.8 L
Isolated Diol	400 mg (65 %)	1.74 g (71%)
Recovered SM	75 mg	145 mg
Adjusted Yield	77 %	77 %

In another fermentation run the amount of starting material was reduced that lead to full conversion of substrate and improved the yield of dihydrodiols. This indicated that higher concentration of substrate acted as limiting factor on the efficiency of growing cells.

	NDO 1	NDO 2
Substrate Acceptance	350 mg/1.8 L	1000 mg/1.8 L
Isolated Yield	80%	75-80%

4.5.5 Fermentation of isoquinoline-3-carbonitrile (3d)

Large scale fermentation was performed using benchtop fermenter according to GP8 (with NDO1) and GP9 (with NDO2) respectively.

Table 4-9 isoquinoline-3-carbonitrile with NDO1 and NDO2 in 2L Fermenter

	NDO 1	NDO 2
Substrate Acceptance	275 mg L ⁻¹	750 mg L ⁻¹
Isolated Yield (overall)	85 %	75-80 %
Product Ratio	90:10	85:15

4.5.6 Comparison of dioxygenases

As far as comparative action of dioxygenases under different condition is concerned, a general trend was observed regarding their activities. The amount of substrate transformed was dependent on number of factors: including amount of substrate added, OD of the growing cells, induction time and agent, solubility of substrate in the aqueous medium, temperature, and duration of experiment. In general TDO was relatively low performing in dihydroxylation of naphthalenes, quinolines and isoquinolines. NDO2 with salicylic acid induction gave best results with isoquinoline-1-carbonitrile **3b** being exception. NDO2 gave best results and showed relatively higher conversions of substrates as compared to NDO1 and TDO. NDO1 showed substrate acceptance and activities in the mid-range and gave better results with isoquinoline-1-carbonitrile **3b** but at rather low concentration of 200 mg L⁻¹.

4.6 Proof of absolute configuration

4.6.1 Reduction of diols

Dihydrodiols produced after *cis*-dihydroxylation of dioxygenases are prone to rearomatization with the loss of water and special care should be employed in order to handle and store them. There are also reports for some of the dihydrodiols having hazardous nature and exploding upon vacuum drying. Hudlicky¹⁰¹ reported the *cis*-diol from β -bromobenzene to be explosive when stored in solid form even at -78°C . The aromatization is exothermic process catalyzed by the trace amounts of phenol. Isolation, purification, and handling of amounts over 5-10 grams must be performed with care. EtOAc extracts must be washed with saturated carbonate solution to remove any trace amounts of phenol which may catalyze the aromatization process.

During our studies of dihydrodiols, we have handled up to 5g of purified products in solid state and have not observed any problems of that kind. However, using dihydrodiols directly for further derivatization steps resulted in aromatization. This proved the previous literature^{194,195,196} of instability of these diols under reaction conditions and hence the need to reduce the adjacent double bond in order to achieve stability and do further reactions with the resulting reduced diols.^{197,198}

On the contrary, there are also few examples where derivatives of dihydrodiols are synthesized directly without reduction of the double bond.^{179,199}

Dihydrodiols after purification by MPLC and drying were generally subjected to subsequent hydrogenation in order to prepare tetrahydrodiols. Small scale hydrogenation was performed by using hydrogen filled balloons at room temperature and ambient pressure. Tetrahydrodiols were not resolved from dihydrodiols over TLC during the reaction because there is almost no difference in the *R_f* value. It was possible to monitor conversion through

¹⁹⁴ [194] V. Berberian, C. C. R. Allen, N. D. Sharma, D. R. Boyd and C. Hardacre, *Advanced Synthesis & Catalysis* **2007**, 349, 727-739.

¹⁹⁵ [195] L. Sbircea, N. D. Sharma, W. Clegg, R. W. Harrington, P. N. Horton, M. B. Hursthouse, D. C. Apperley, D. R. Boyd and S. L. James, *Chemical Communications* **2008**, 5538-5540.

¹⁹⁶ [196] D. R. Boyd, N. D. Sharma, M. V. Berberian, K. S. Dunne, C. Hardacre, M. Kaik, B. Kelly, J. F. Malone, S. T. McGregor and P. J. Stevenson, *Advanced Synthesis & Catalysis* **2010**, 352, 855-868.

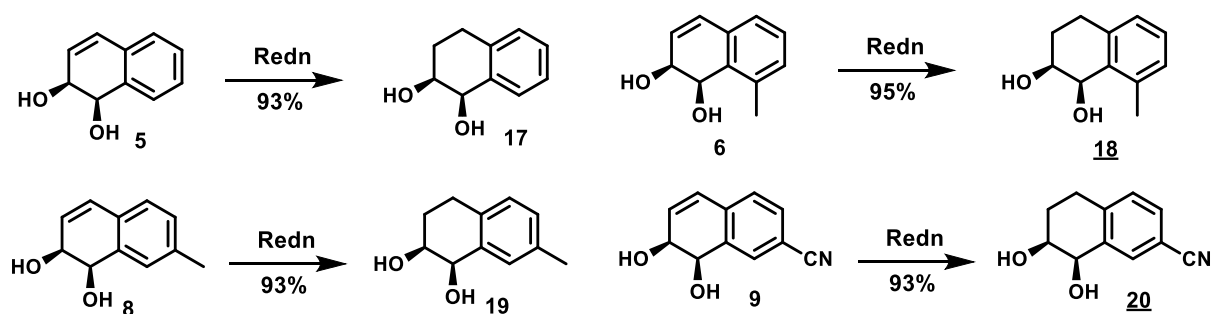
¹⁹⁷ [197] M. E. Lagreca, I. Carrera, G. A. Seoane and M. Brovotto, *Tetrahedron Lett.* **2014**, 55, 853-856.

¹⁹⁸ [198] D. R. Boyd, N. D. Sharma, I. N. Brannigan, T. A. Evans, S. A. Haughey, B. T. McMurray, J. F. Malone, P. B. A. McIntyre, P. J. Stevenson and C. C. R. Allen, *Organic & Biomolecular Chemistry* **2012**, 10, 7292-7304.

¹⁹⁹ [199] D. R. Boyd, N. D. Sharma, N. M. Llamas, C. R. O'Dowd and C. C. R. Allen, *Organic & Biomolecular Chemistry* **2006**, 4, 2208-2217.

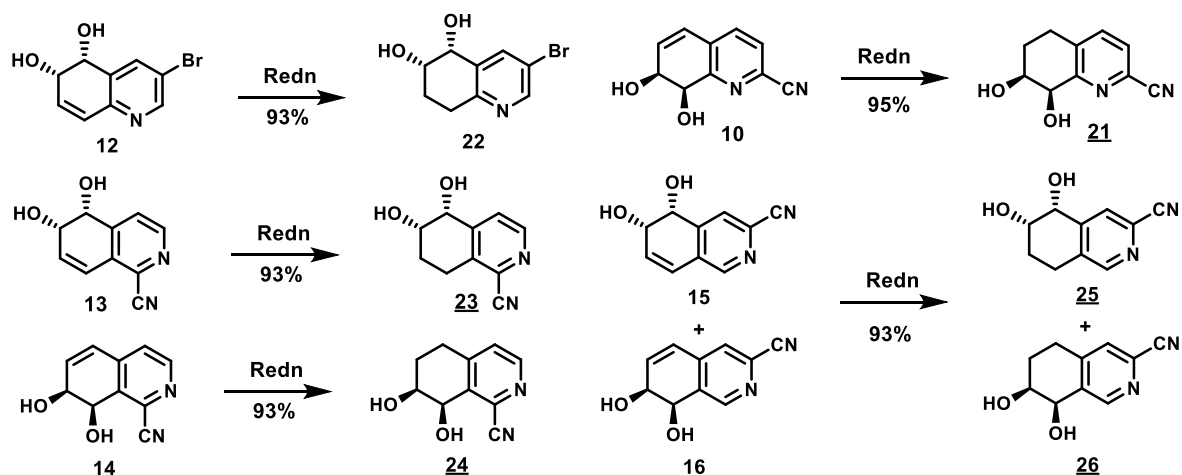
GCMS analysis of aliquots as increase in the mass value by 2 due to addition of 2H atoms to the substrate could be resolved. After completion of reaction, tetrahydrodiols were obtained in analytical purity after passing through small bed of Celite[®] and eluting by EtOAc over small column of silica gel. Few more examples from literature about reduction of diols to increase the stability and further applications can be found here.^{200, 201, 202, 203}

Scheme 4-1 gives details about tetrahydrodiols prepared by catalytic hydrogenation of naphthalene and substituted naphthalene dihydrodiols.



Scheme 4-1 Catalytic hydrogenation of different naphthalene diols

Likewise, dihydrodiols obtained by biotransformation of quinoline and isoquinoline substrates were reduced. Scheme 4-2 gives details about tetrahydrodiols prepared by catalytic hydrogenation of quinoline and isoquinoline diols.



Scheme 4-2 Catalytic hydrogenation of quinoline and isoquinoline diols

²⁰⁰ [200] F. M. Hauser and S. Prasanna, *Synthesis* **1980**, 1980, 621-623.

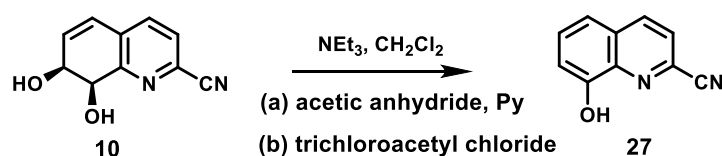
²⁰¹ [201] L. Emmanuvel, T. M. A. Shaikh and A. Sudalai, *Organic Letters* **2005**, 7, 5071-5074.

²⁰² [202] D. R. Boyd, N. D. Sharma, R. Boyle, R. A. S. McMordie, J. Chima and H. Dalton, *Tetrahedron Letters* **1992**, 33, 1241-1244.

²⁰³ [203] D. R. Boyd, N. D. Sharma, R. Agarwal, N. A. Kerley, R. A. S. McMordie, A. Smith, H. Dalton, A. J. Blacker and G. N. Sheldrake, *Journal of the Chemical Society, Chemical Communications* **1994**, 1693-1694.

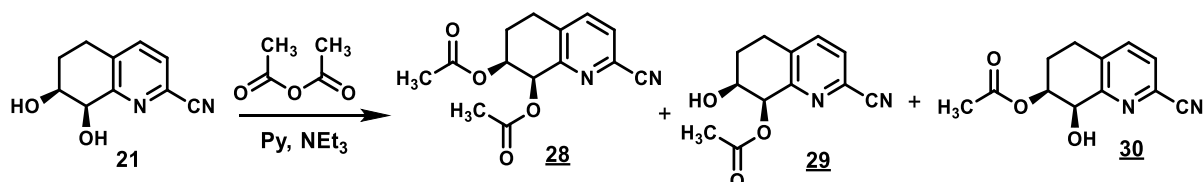
4.6.2 Synthesis of acetate esters

In order to prove the absolute structure of dihydrodiols formed by dioxygenases attempts were made to synthesize the ester derivatives. The ester derivatives could be then crystallized and with the X-ray crystallographic analysis the absolute configuration of corresponding diol could be established. In order to synthesize the acetate esters of reduced diols, dihydrodiol **10** was reacted with acetic anhydride in pyridine using triethylamine as base. This resulted in 8-hydroxy quinoline-2-carbonitrile (**27**) instead of acetate ester as product of elimination of water and subsequent rearomatization. When reacted with trichloroacetyl chloride, it again resulted in aromatized product **27** that was confirmed by NMR analysis of crude product of the reaction.



Scheme 4-3 Reaction of unsaturated diol resulted in elimination to compound **27**

After attempts to synthesize acetate esters with dihydrodiol failed, it was subjected to catalytic hydrogenation using Pd on activated charcoal. The reaction of tetrahydrodiol **21** with acetic anhydride in pyridine as solvent and NEt_3 as base underwent without any problems and diacetate ester **28** was produced in overnight. The reaction was also performed with small amount of DMAP and presence or absence of DMAP did not have any effect on yield of reaction. Pyridine itself is enough to work as solvent and base for acetylation reaction. The reaction worked also fine in dry DCM as solvent and an excess of NEt_3 (3-5 equivalents) as base. The esterification reactions were carried out in dry DCM and NEt_3 as base. The reaction works fine and the addition of base should be done slowly and at lower temperature.



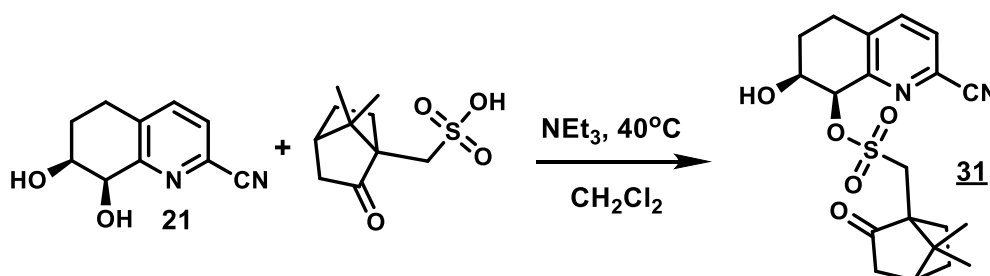
Scheme 4-4 Synthesis of mono and diacetate esters

The diacetate ester **28** could not be crystallized using methanol, ethanol or EtOAc as solvent; an attempt was made to synthesize and isolate monoester of tetrahydrodiol **21** and try to crystallize it. In order to synthesize monoester, the reaction was carried out at low

temperature under dilute conditions and quenched after 3 hrs, with all three derivatives present in the reaction mixture. When left longer to react or temperature let to rise above room temperature, the percentage of diester increased.

4.6.3 Synthesis of camphorsulfonyl esters

In order to synthesize the camphorsulfonyl derivative of tetrahydrodiol **21**, it was reacted with camphorsulfonic acid in DCM using NEt_3 as base. The reaction was not complete at room temperature overnight and it was necessary to heat it to 40°C . After column chromatography a single product was isolated being monoester of 8-OH. Monoester **31** did not afford suitable crystals for X-ray analysis being gummy like substance.



Scheme 4-5 Synthesis of camphorsulfonyl esters

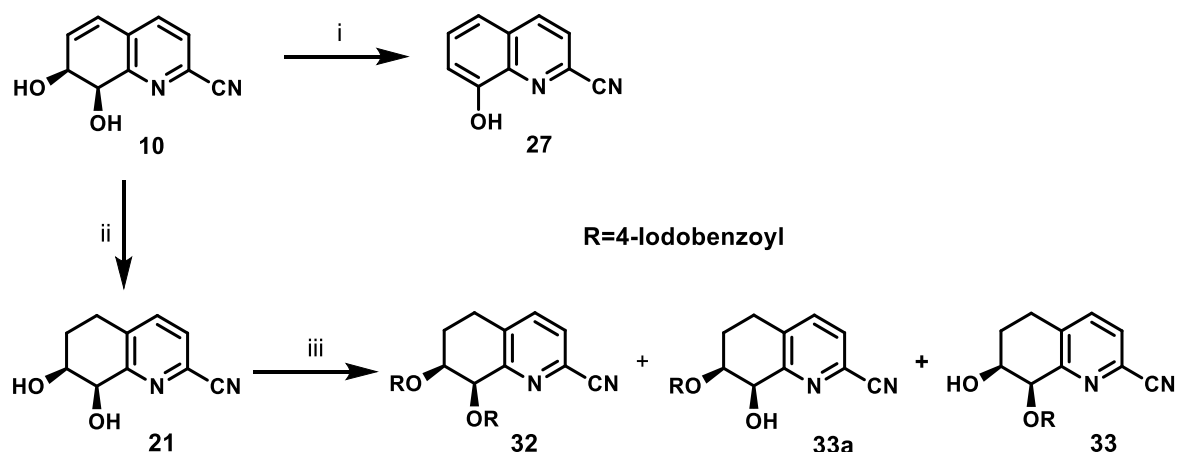
4.6.4 Synthesis of 4-iodobenzoate esters

Since no suitable crystals were formed from the camphorsulfonate ester, an alternative method was employed in order to prove the absolute configuration of diol. The idea was to synthesize a derivative of the $-\text{OH}$ group containing heavy halogen atom and then try to crystallize it. If suitable crystals from the derivative formed, X-ray analysis could provide better picture in order to establish the absolute configuration of the diols. In order to do this, reduced diol **21** was reacted with 4-iodobenzoyl chloride to synthesize 4-iodobenzoate esters. As observed in the cases of acetate and camphorsulfonate esters that both $-\text{OH}$ groups show difference in the reactivity, it was expected that mixture of mono and diesters would be formed. This could give more options to see which of the products produce suitable crystals for X-ray analysis.

First, tetrahydrodiol **21** was reacted with excess of 4-iodobenzoyl chloride (2.2 eq) in order to produce only diester **32**. After purification, different methods were tried to get crystals of diester **32** including different solvent combinations (EtOAc, PE, MeOH, EtOH, diethyl ether, diisopropyl ether) at room temperature and also at lower temperatures in the

freezer at -20°C . It always gave plain solid compound after solvent evaporation which behaved like glass or super cooled liquid and upon heating liquefied at 95°C . As the diester couldn't give suitable crystals, efforts were made to prepare and isolate the corresponding monoester of the tetrahydrodiol. It was reacted with small excess of 4-iodobenzoyl chloride (1.1 eq) and larger amount of solvent and reaction was not run to absolute completion but rather stopped when TLC showed considerable presence of monoester in the reaction mixture. All the products and unreacted diol were separated by flash chromatography by using MPLC. Monoester **33a** at 7-OH was formed in traces whereas monoester **33** at 8-OH was formed at higher ratio indication relatively higher reactivity of 8-OH position of tetrahydrodiol **21**.

Monoester **33** of tetrahydrodiol **21** afforded nice crystals and could be submitted for X-ray analysis. Scheme 4-5 shows details about the ester synthesis with 4-iodobenzoyl chloride.



Scheme 4-6 Reagents: i- DCM, NEt_3 , 4-iodobenzoyl chloride,; ii- H_2 , 10 % Pd (C),; iii- NEt_3 , DCM, 4-iodobenzoyl chloride.

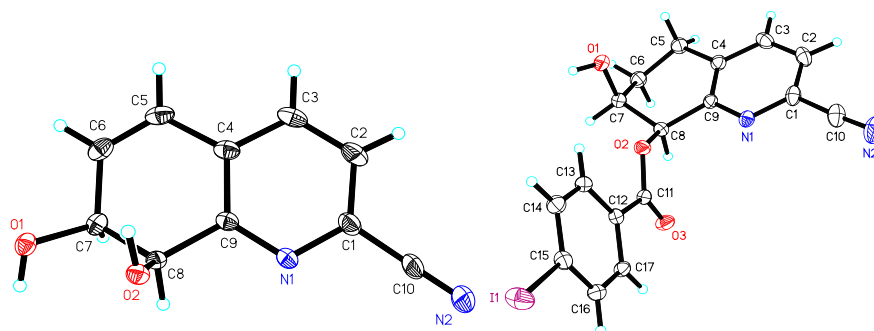
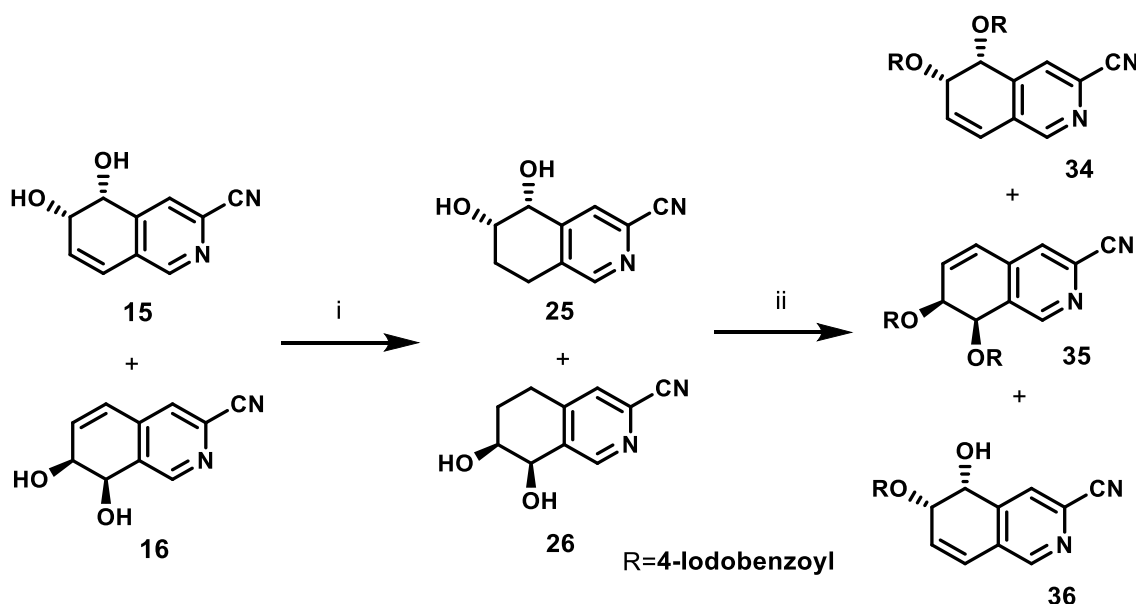


Fig. 4-3 X-ray crystal structures of diol **21** and monoester **33**.

Similar strategy was applied in order to prove the absolute configuration of diols produced by biotransformation of isoquinoline-3-carbonitrile **3d**. Since two dihydrodiols were produced after biotransformation of isoquinoline-3-carbonitrile, and could not be separated successfully, attempts were made to derivatize them in mixture and try to separate them afterwards. An 85:15 mixture of diols **15** and **16** was reduced to the mixture of tetrahydrodiols **25** and **26** respectively. Then, the mixture of tetrahydro diols was reacted with 4-iodobenzoyl chloride and products were separated by chromatography. First least polar fraction isolated was identified as diester **35** of corresponding minor diol **26**. Diester **35** was only compound that corresponded to the otherwise, non-isolable 7,8-diol **16**. Major tetrahydro diol **25** gave mixture of diester **34** and monoester **36** at 6-OH. Monoester at 5-OH was not formed in isolable quantities. Diester **34** behaved exactly similarly as diester **32** of quinoline-2-carbonitrile in terms of crystallization and also in terms of melting point. It had even same melting point as that of diester **32** (95 °C). Monoester **36**, however, gave fine crystals which were analyzed by X-ray crystallography in order to prove the absolute configuration of corresponding diol, and was confirmed as *cis*-(5*R*,6*S*)-3-cyano-5-hydroxy-5,6,7,8-tetrahydro isoquinolin-6-yl-4-iodobenzoate. Scheme 4-6 shows details about the ester synthesis with 4-iodobenzoyl chloride. Fig. 4-3 shows the X-ray crystal structures of diol **15** and monoester **36**.



Scheme 4-7 Reagents: i- H₂, 10 % Pd/C; ii- 4-iodobenzoyl chloride, NEt₃, DCM.

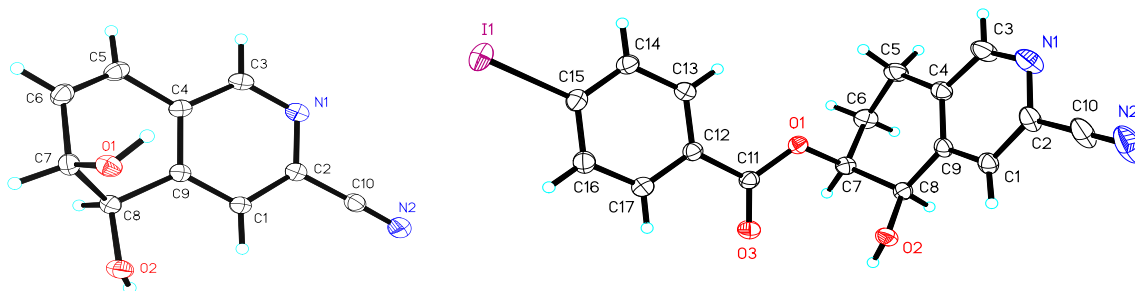
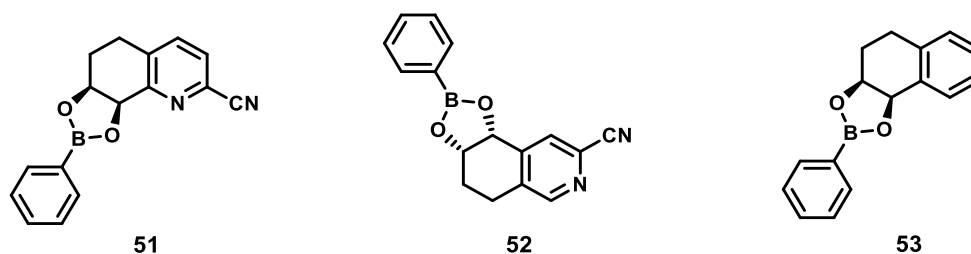


Fig. 4-4 X-ray crystal structures of diol **15** and monoester **36**.

4.6.5 Synthesis of boronic esters

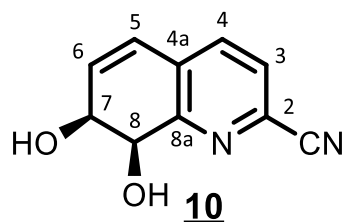
Another approach to derivatize the *cis*-diols obtained by dihydroxylation to corresponding phenyl boronic esters has been reported. The resulting phenyl boronic esters could then be analyzed by NMR and crystallographic studies in order to prove the absolute configuration of corresponding *cis*-diols. The synthesis of phenyl boronic esters was relatively trivial and easily accessible. Boronic esters of tetrahydrodiols were synthesized by reacting with phenyl boronic acid at room temperature and at atmospheric pressure by mixing them together in dry DCM.



4.7 Structure assignment of products

4.7.1 Structure assignment of diol **10**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Starting from saturated compound **2a** generation of two new stereogenic centers as a result of biocatalytic dihydroxylation to diol **10** was scrutinized. One would expect three sets of signals for the ^1H NMR spectra of which H7 and H8 being attached to C with OH groups, H5 and H6 being olefinic protons and H3 and H4 being aromatic protons. ^{13}C signals of C atoms would also follow the same pattern with an additional set of quaternary carbons being C2, C4a, C8a, and CN. Based on ^1H - ^{13}C correlations it was found that H8 (δ 4.50 ppm) was directly attached to C8 (δ 71.2 ppm), H7 (δ 4.41 ppm) with C7 (δ 68.0 ppm), H6 (δ 6.24 ppm) with C6 (δ 137.3 ppm), H5 (δ 6.59 ppm) with C5 (δ 124.5 ppm), H4 (δ 7.55 ppm) with C4 (δ 134.4 ppm), H3 (δ 7.91 ppm) with C3 (δ 129.2 ppm). ^1H - ^1H correlation confirmed vicinal spin network of H8 with H7, H7 with H6 and H8, H6 with H5 and H7, and H4 with H3. In DMSO the coupling of OH-7 with H7 and OH-8 with H8 was also observed. HMBC spectra showed long-range coupling C8a with H8, H5, and H4 respectively. C4a showed long range coupling with H3 and H6 and also with H5 and H8. CN showed coupling with H3 and H4. The crystal structure analysis also showed that the sample was enantiopure and had a 7*S*, 8*R* configuration and identified as *cis*-(7*S*,8*R*)-7,8-dihydroxy-7,8-dihydroquinoline-2-carbonitrile (**10**).

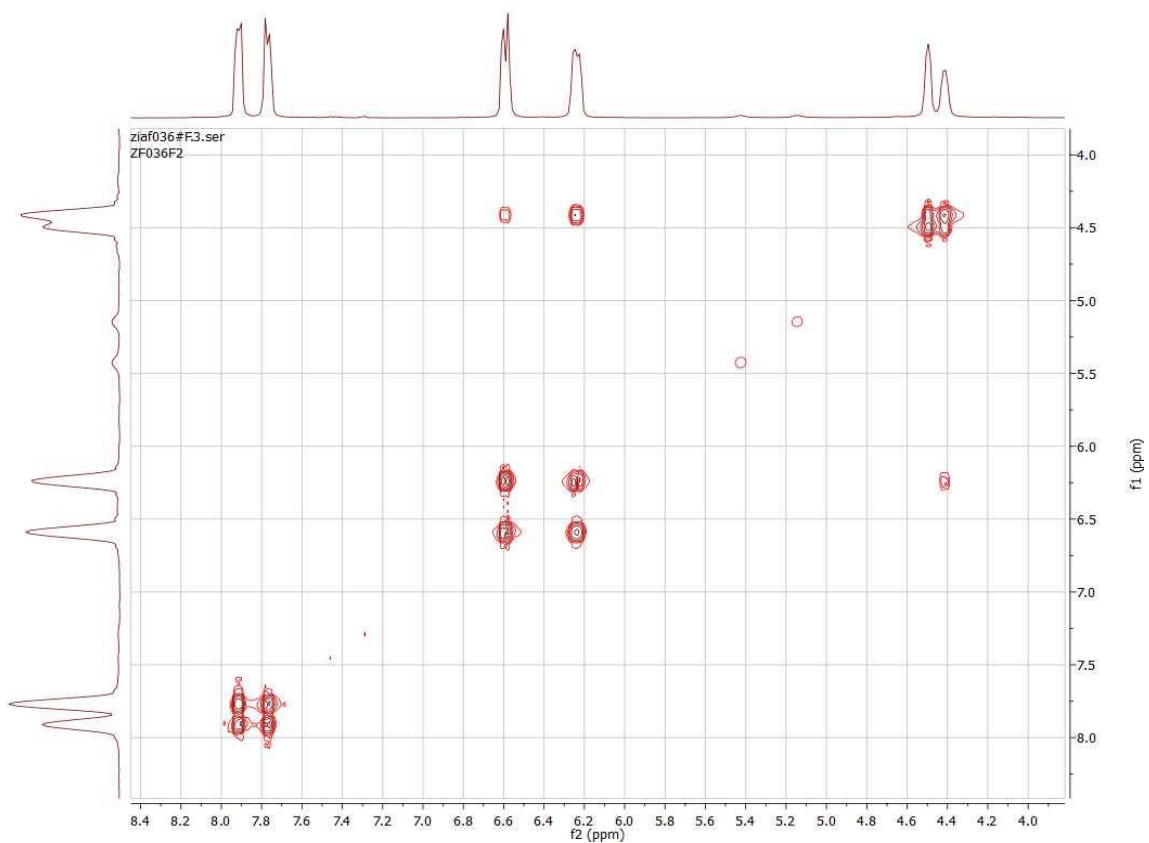


Fig. 4-5 H,H-Cosy spectral data for diol **10**

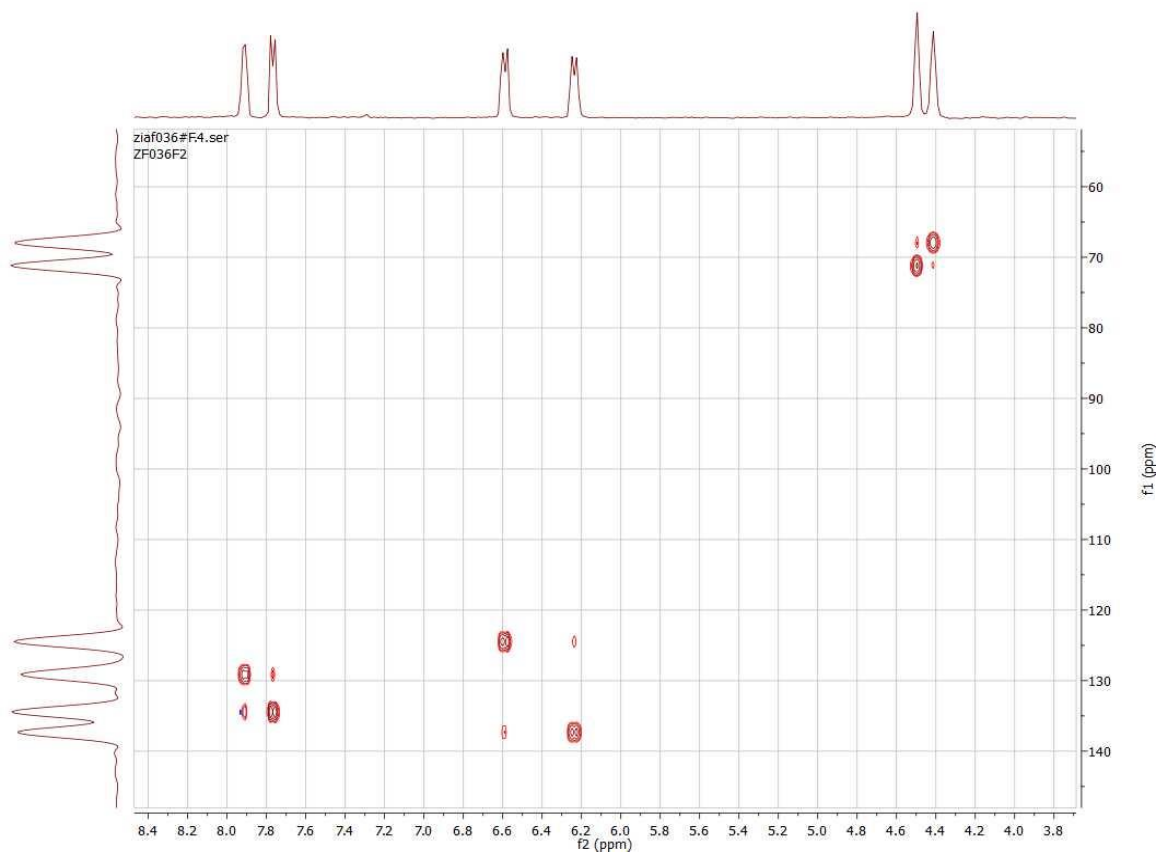


Fig. 4-6 HSQC spectral data for diol **10**

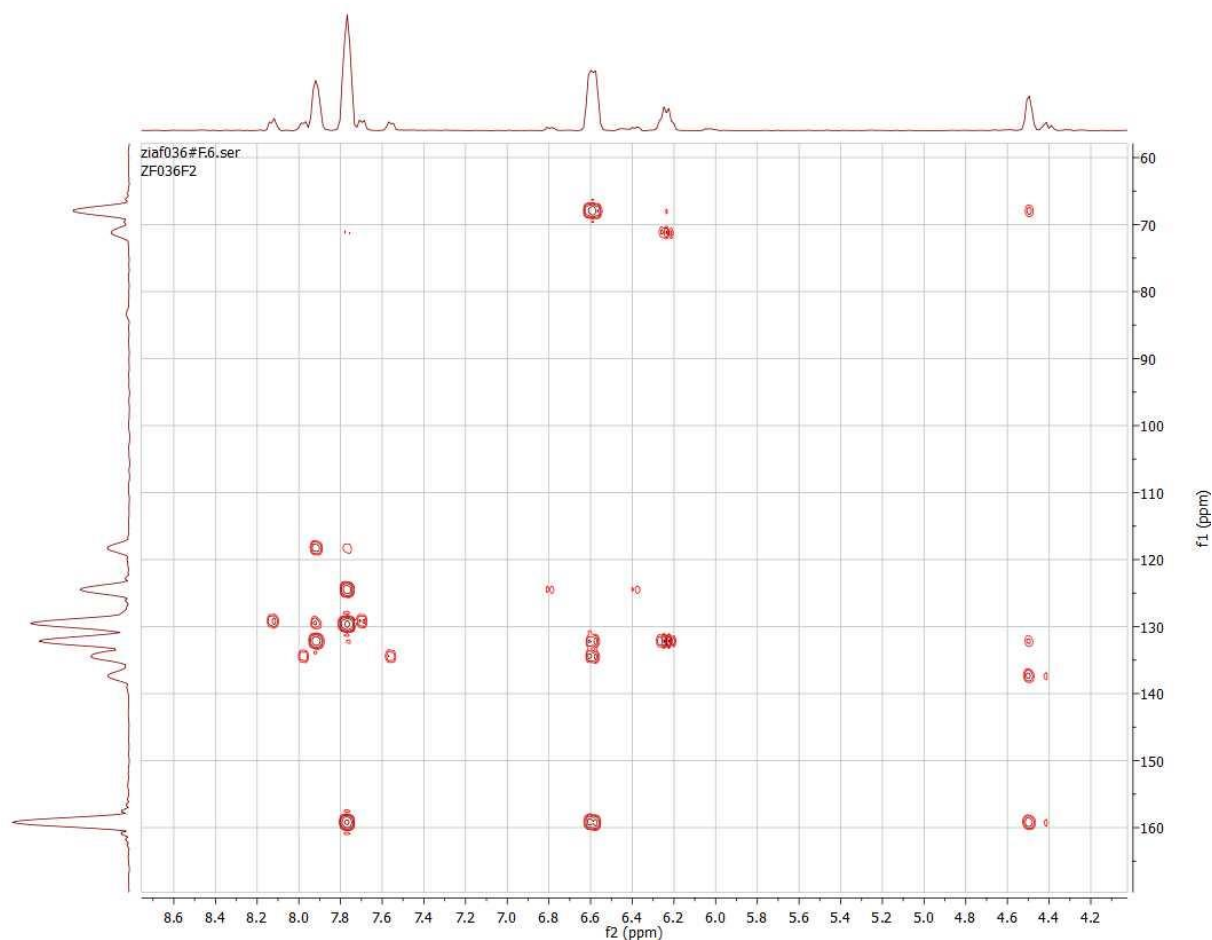
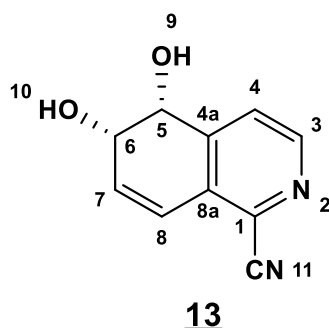


Fig. 4-7 HMBC spectral data for diol **10**

4.7.2 Structure assignment of diol **13**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Starting from saturated isoquinoline-1-carbonitrile (**3b**) generation of two new stereogenic centers as a result of biocatalytic dihydroxylation to diol **13** was scrutinized. Three sets of signals for the ^1H NMR spectra for H5 and H6 attached to C5 and C6 with OH groups, H7 and H8 being olefinic protons and H3 and H4 being aromatic protons, were

expected. ^1H - ^1H correlation confirmed vicinal spin network of H5 with H6, H6 with H5 and H7, H7 in the vicinity of H6 and H8, and H4 with H3. ^{13}C signals of C atoms would also follow the same pattern with an additional set of quaternary carbons being C1, C4a, C8a, and CN. Based on ^1H - ^{13}C correlations it was found that H6 (δ 4.29 ppm) was directly attached to C6 (δ 66.2 ppm), H5 (δ 4.73 ppm) with C5 (δ 70.6 ppm), H7 (δ 6.55 ppm) with C7 (δ 136.1 ppm), H8 (δ 6.89 ppm) with C8 (δ 123.7 ppm), H4 (δ 7.82 ppm) with C4 (δ 125.7 ppm), and H3 (δ 8.55 ppm) with C3 (δ 151.2 ppm). H HMBC spectra showed long-range coupling of C8a with H5, H4 and considerable coupling with H3 and H6 respectively. C4a showed long range coupling with H3, H6 and H8.

The crystal structure analysis also showed that the sample was enantiopure and had a *5R,6S* configuration and identified as *cis*-(5*R*,6*S*)-5,6-Dihydroxy-5,6-dihydroisoquinoline-1-carbonitrile (**13**).

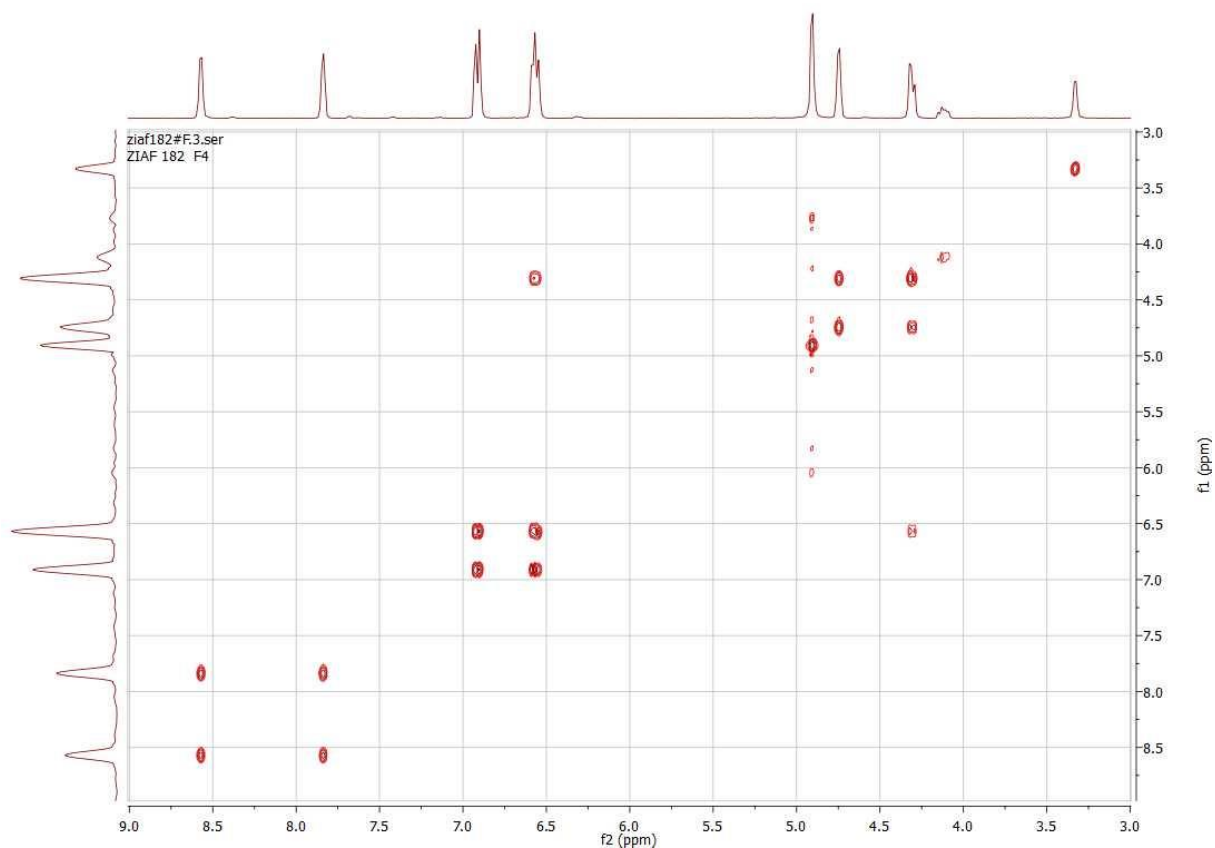


Fig. 4-8 H,H-Cosy spectral data for diol **13**

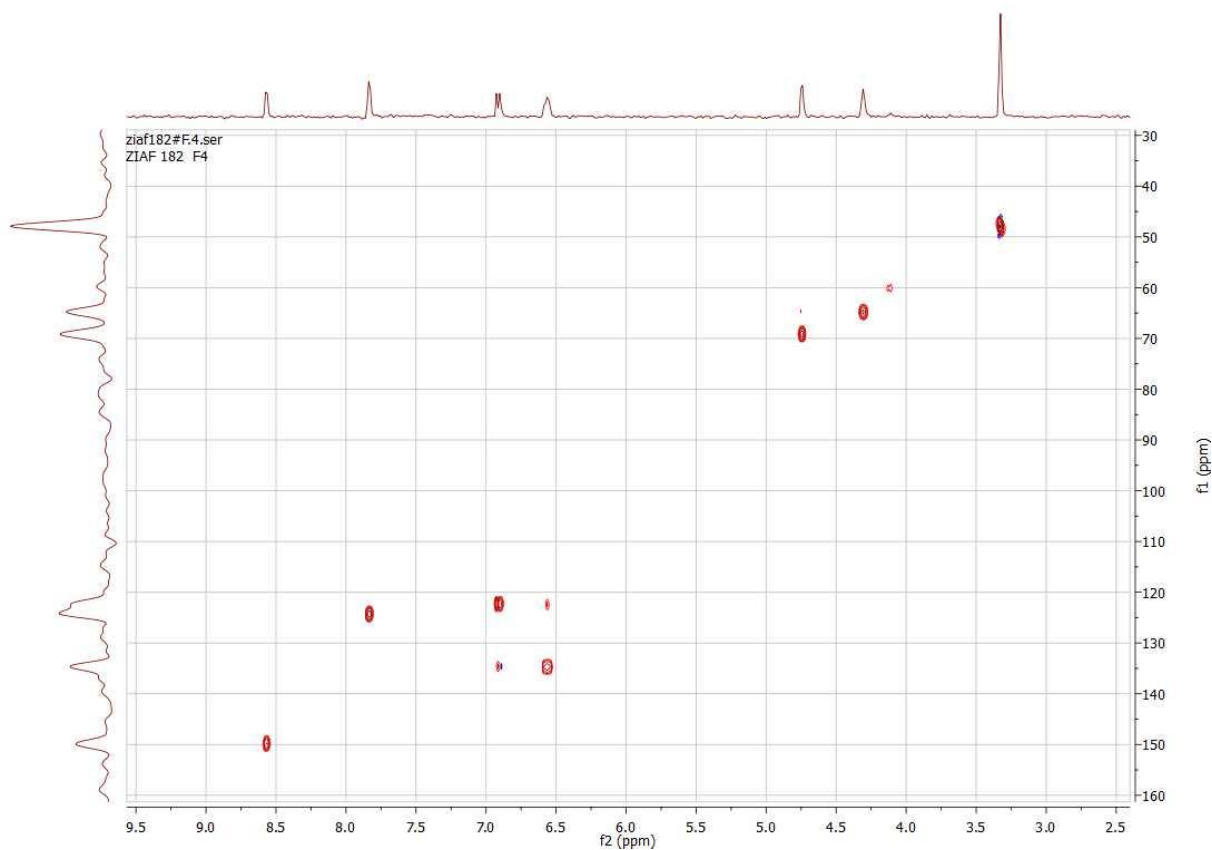


Fig. 4-9 HSQC spectral data for diol **13**

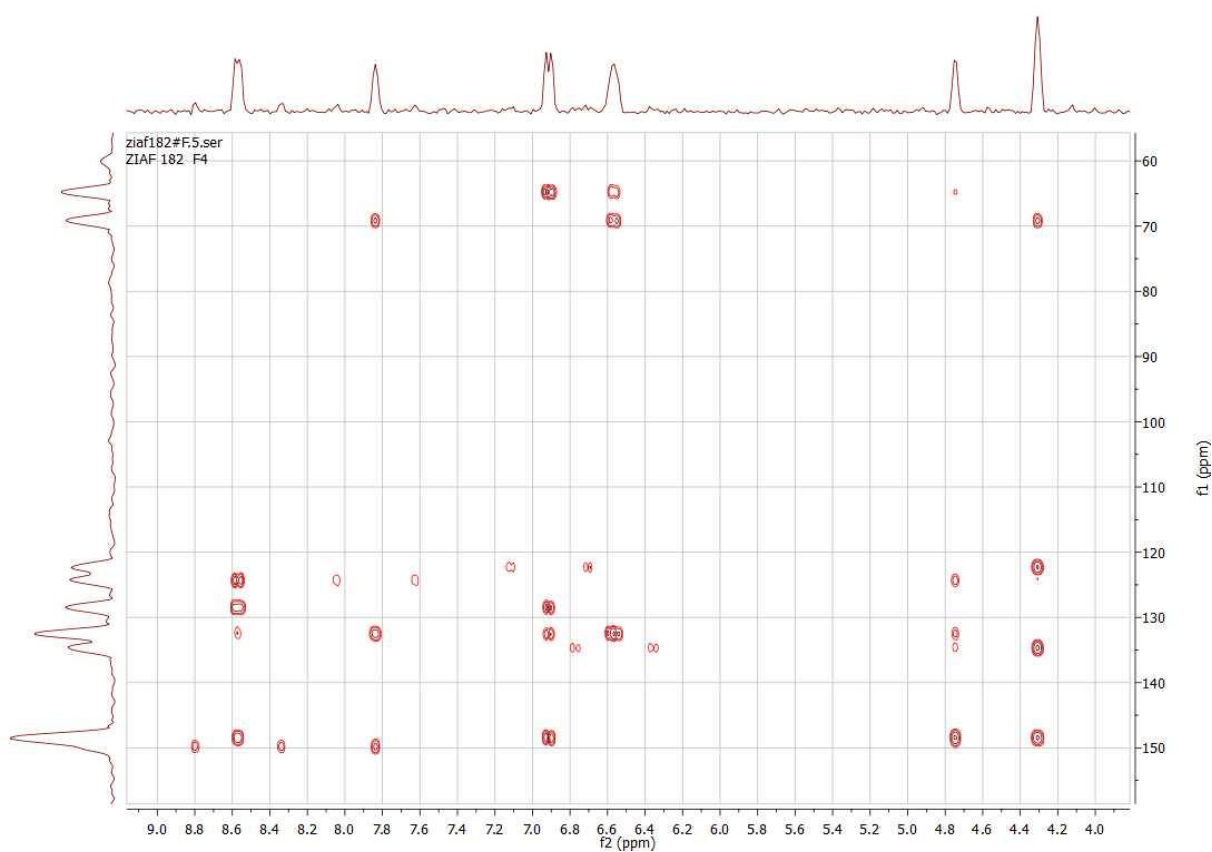
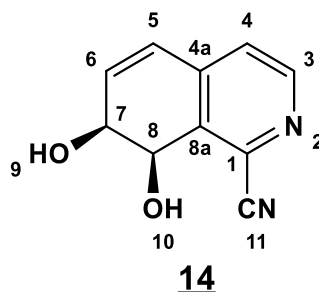


Fig. 4-10 HMBC spectral data for diol **13**

4.7.3 Structure assignment of diol **14**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Following the same pattern of diol **10** and **13**, three sets of signals for the ^1H NMR spectra for H7 and H8 attached to C7 and C8 with OH groups, H5 and H6 being olefinic protons and H3 and H4 being aromatic protons, were expected. ^{13}C signals of C atoms would also follow the same pattern with an additional set of quaternary carbons being C1, C4a, C8a, and CN. ^1H - ^1H correlation confirmed vicinal spin network of H8 with H7, H7 with H6 and H8, H6 with H5 and H7, and H4 with H3. Based on ^1H - ^{13}C correlations it was found that H8 (δ 4.91 ppm) was directly attached to C8 (δ 67.4 ppm), H7 (δ 4.57 ppm) with C7 (δ 70.3 ppm), H6 (δ 6.29 ppm) with C6 (δ 140.8 ppm), H5 (δ 6.55 ppm) with C5 (δ 124.7 ppm), H4 (δ 7.40 ppm) with C4 (δ 125.1 ppm), H3 (δ 8.56 ppm) with C3 (δ 152.6 ppm) which was in accordance with the cross-peaks.

HMBC spectra showed long-range coupling of H8 with C7 and C8a over two bonds distance ($^2J_{\text{HC}}$) and with C1, C4a, and C7 over three bonds distance ($^3J_{\text{HC}}$). H7 showed two bonds coupling ($^2J_{\text{HC}}$) with C8a and C6 and also showed four bonds coupling with C4 ($^4J_{\text{HC}}$). H6 showed long range coupling with C8, C8a, and C4a. H5 showed long range coupling with C4, C7, C4a, and C8a. H4 showed long range coupling with C3, C5 and C8a. H3 showed long range coupling with C1, C4a, C8a, and C4.

The crystal structure analysis also showed that the sample was enantiopure and had a 7S,8R configuration and identified as *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-1-carbonitrile (**14**).

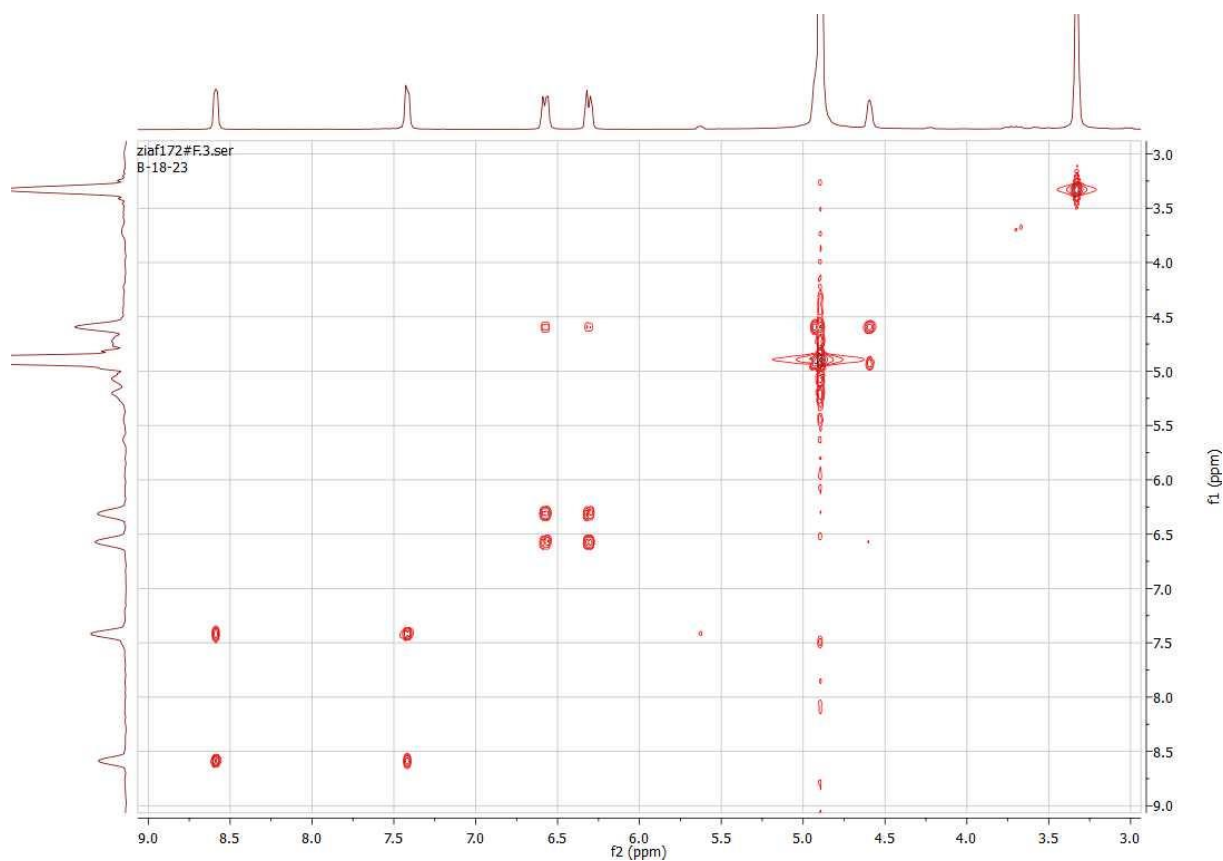


Fig. 4-11 H,H-Cosy spectral data for diol **14**

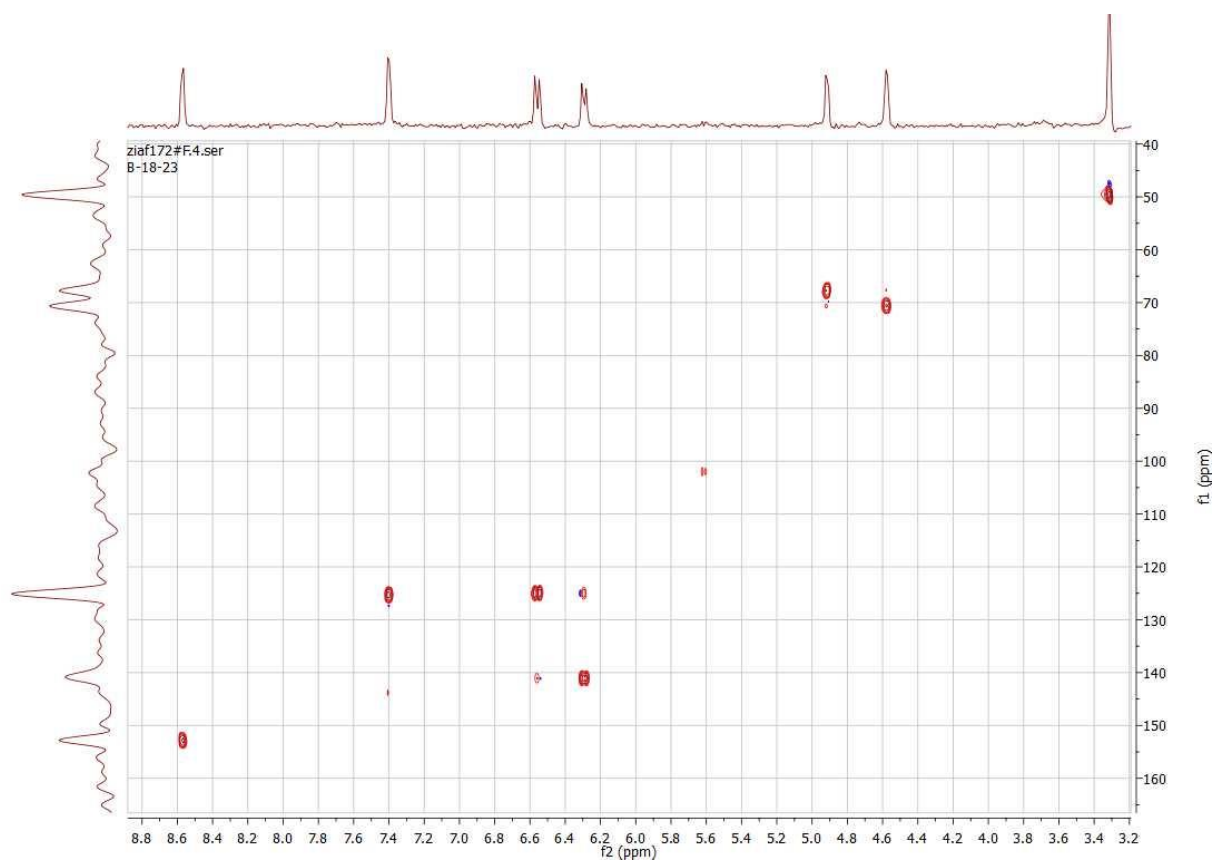


Fig. 4-12 HSQC spectral data for diol **14**

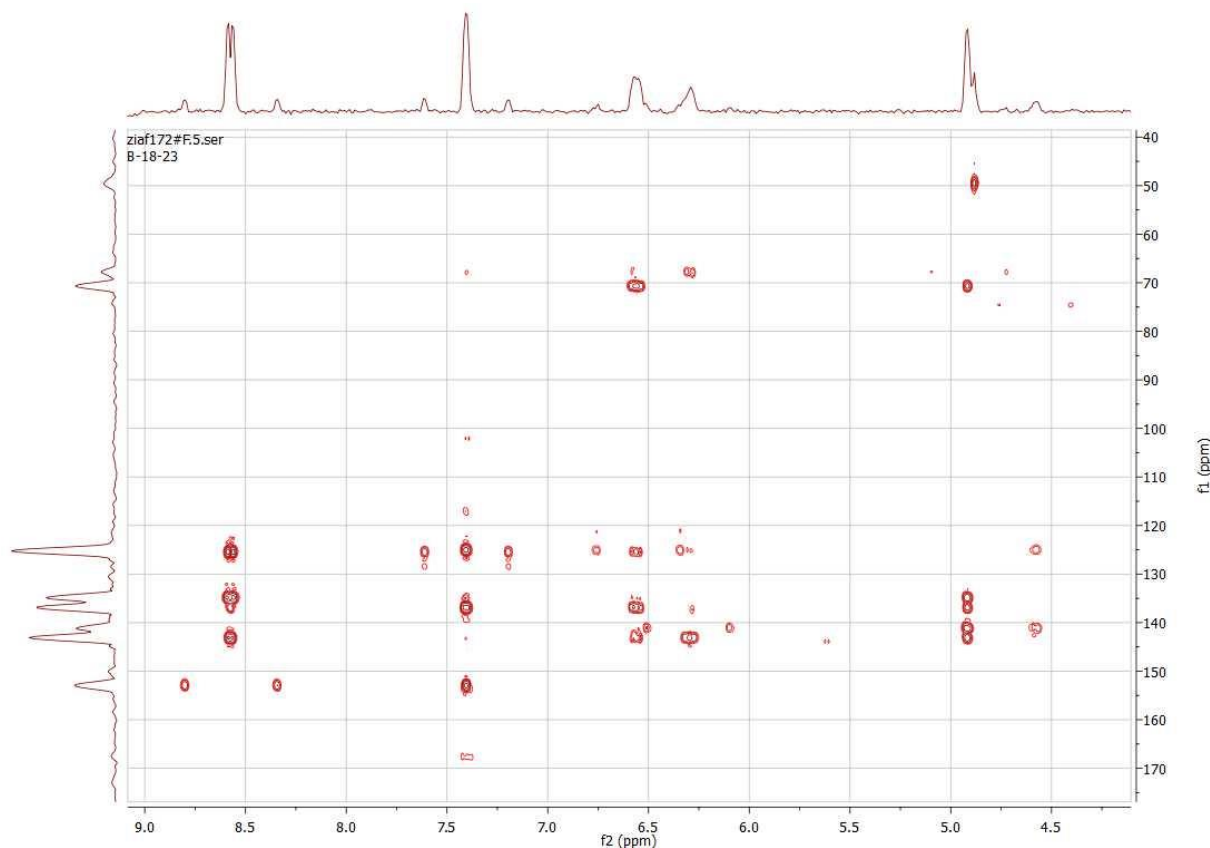
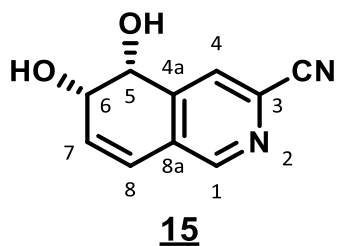


Fig. 4-13 HMBC spectral data for diol **14**

4.7.4 Structure assignment of diol **15**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Starting from saturated isoquinoline-3-carbonitrile (**3d**) formation of two new stereogenic centers as a result of biocatalytic dihydroxylation to diol **15** was scrutinized.

Three sets of signals for the ^1H NMR spectra of which H5 and H6 being attached to C with OH groups, H7 and H8 being olefinic protons and H1 and H4 being aromatic protons. ^{13}C signals of C atoms would also follow the same pattern with an additional set of quaternary carbons being C3, C4a, C8a, and CN.

Based on ^1H - ^{13}C correlations and cross peaks H7 (δ 6.41 ppm) was found to be directly attached to C7 (δ 135.6 ppm), H8 (δ 6.70 ppm) with C8 (δ 124.0 ppm), H4 (δ 7.93 ppm) with C4 (δ 125.9 ppm), and H1 (δ 8.54 ppm) with C1 (δ 148.0 ppm). ^1H - ^1H correlation confirmed vicinal spin coupling of H6 with H5 and H8. ^1H - ^{13}C correlations confirmed H6 (δ 4.15 ppm) with C6 (δ 64.8 ppm) and H5 (δ 4.59 ppm) with C5 (δ 69.3 ppm). H HMBC spectra showed long-range coupling of H8 with C5, C6, C8a and C4a; H7 with C6, C5, C8, C8a, and C4a; H6 with C8, C8a, and C4a; H5 with C6, C4a, C4, C7, C8a, and C3; H4 with C5, C8a, C4a, and CN; and H1 with C8, C5, C4a, C4, and C3.

The crystal structure analysis also showed that the sample was enantiopure and had a 5R,6S configuration and identified as *cis*-(5R,6S)-5,6-Dihydroxy-5,6-dihydroisoquinoline-3-carbonitrile (**15**)

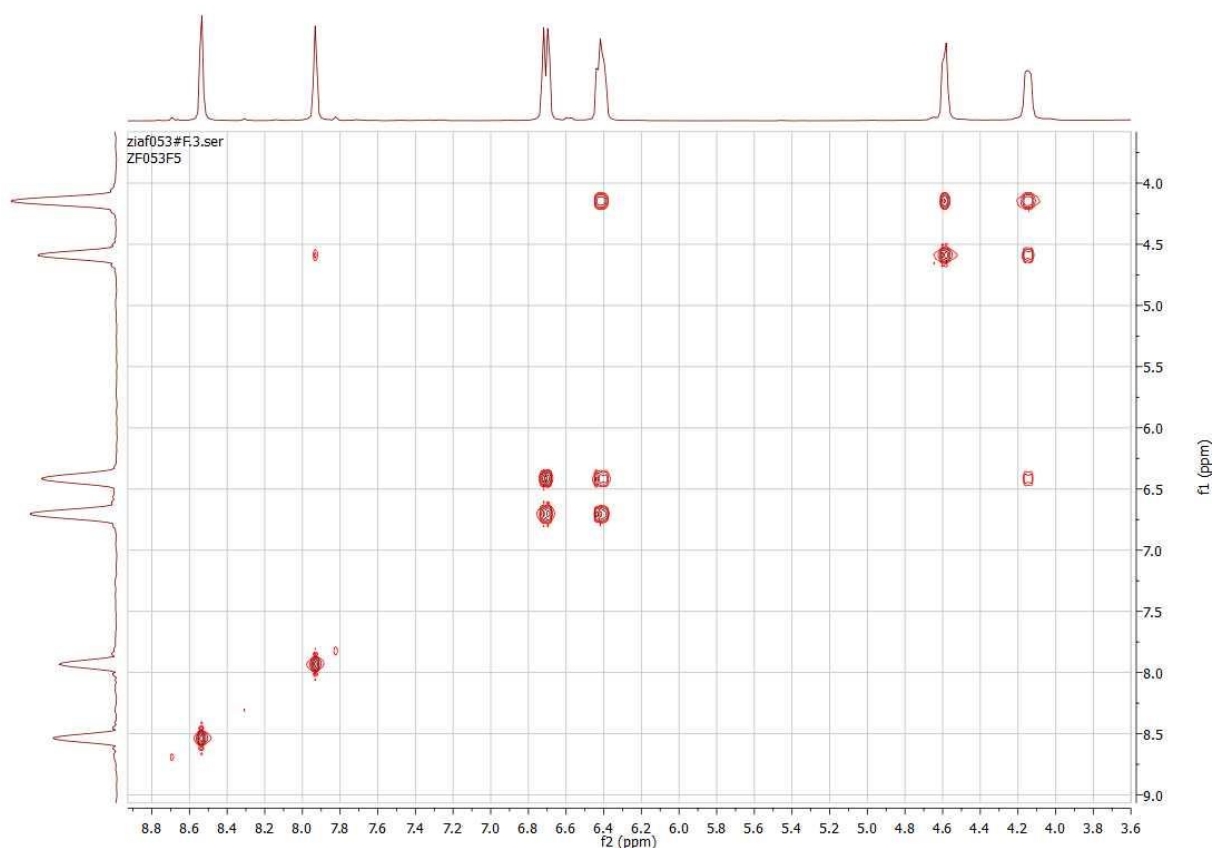


Fig. 4-14 H,H-Cosy spectral data for diol **15**

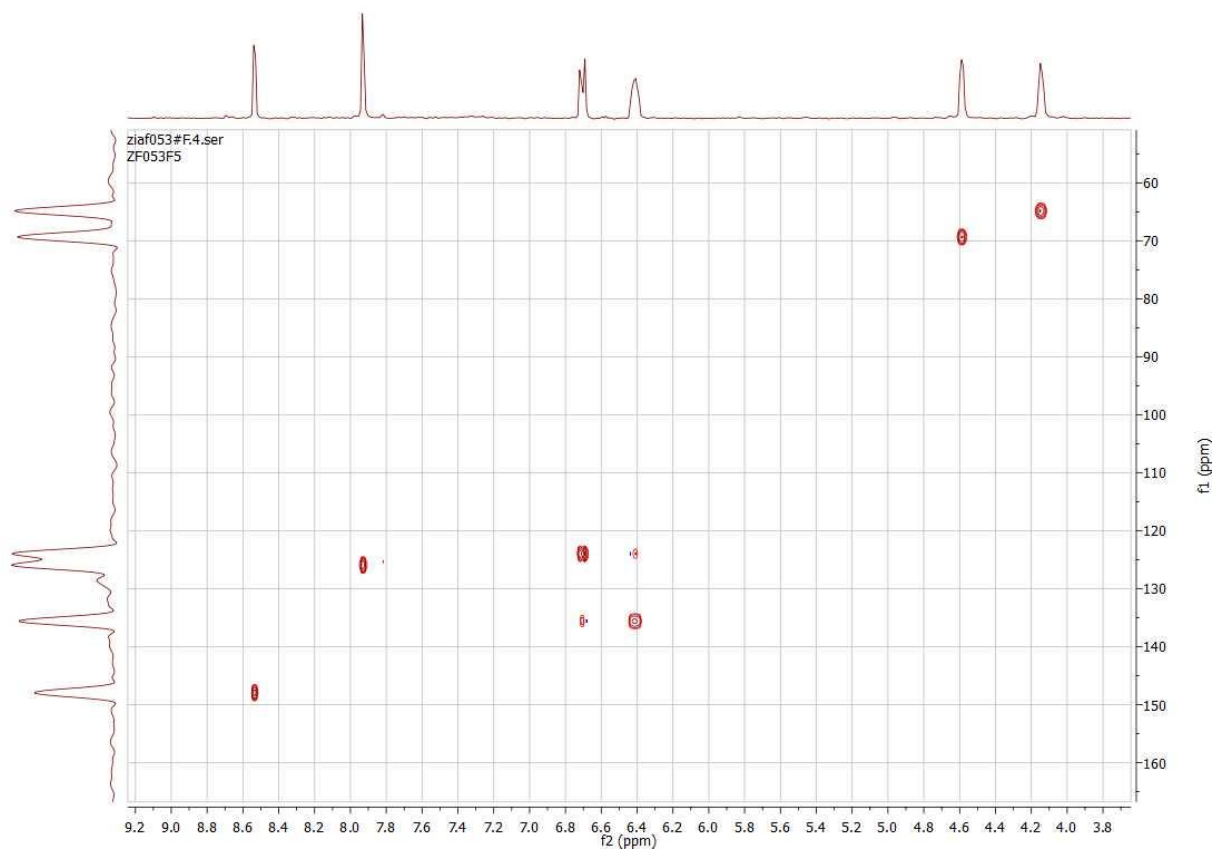


Fig. 4-15 HSQC spectral data for diol **15**

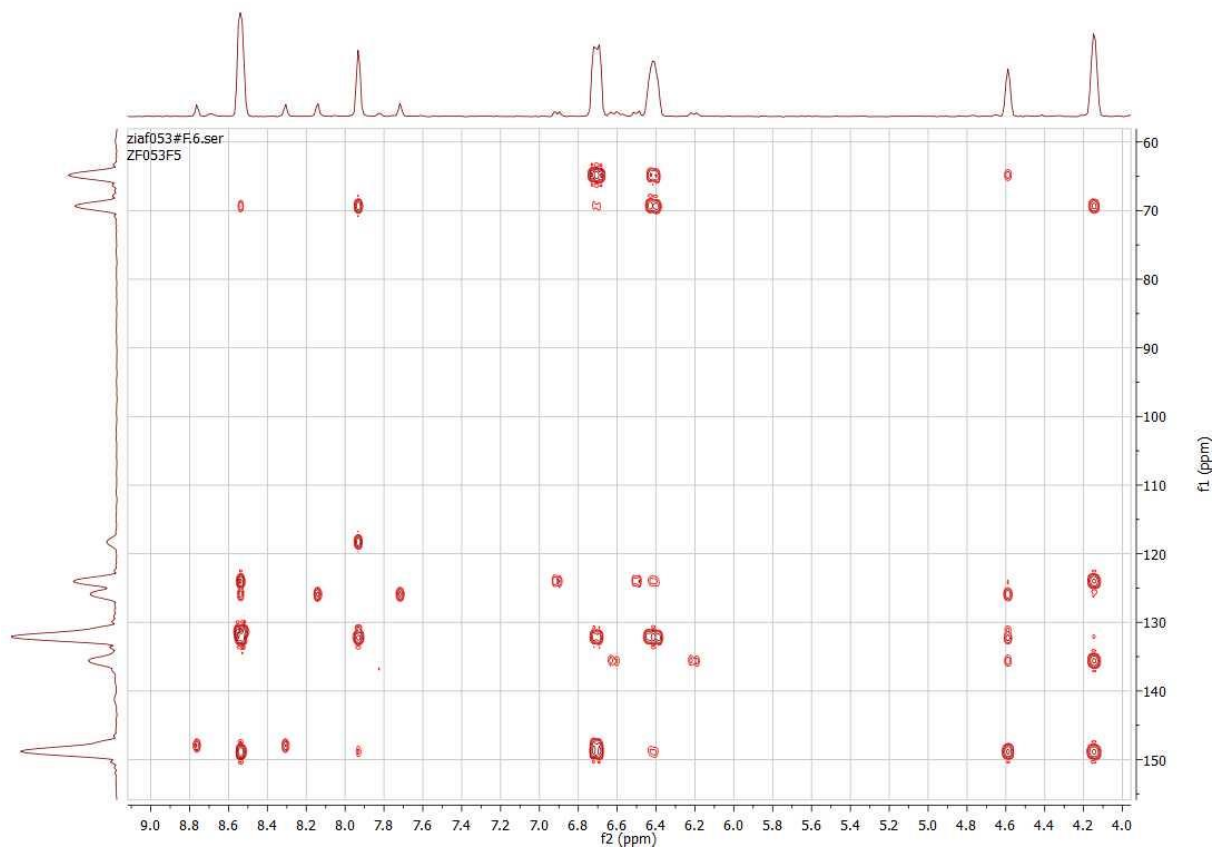
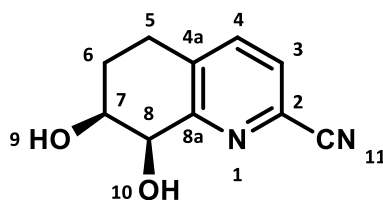


Fig. 4-16 HMBC spectral data for diol **15**

4.7.5 Structure assignment of reduced diol **21**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



21

Starting from corresponding dihydrodiol **10**, structure of tetrahydrodiol **21** was scrutinized. Complete shift of signals for 5 and 6 from olefinic region to aliphatic region was expected. One would expect three sets of signals for the ^1H NMR spectra of which H5 and H6 being aliphatic protons, H7 and H8 being attached to C with OH groups, and H3 and H4 being aromatic protons. ^{13}C signals of C atoms would also follow the same pattern with an additional set of quaternary carbons being C2, C4a, C8a, and CN.

When measured in CDCl_3 , based on ^1H - ^{13}C correlations it was found that H8 (δ 4.64 ppm) was directly attached to C8 (δ 70.6 ppm), H7 (δ 4.41 ppm) with C7 (δ 66.4 ppm), CH_2 -6 (δ 1.96-2.26 ppm) with C6 (δ 25.0 ppm), CH_2 -5 (δ 2.82-3.17 ppm) with C5 (δ 23.8 ppm), H3 (δ 7.55 ppm) with C3 (δ 127.2 ppm), H4 (δ 7.59 ppm) with C4 (δ 137.3 ppm). ^1H - ^1H correlation confirmed vicinal spin network of H8 with H7, H7 with H6 and H8, H6 with H5 and H7, and H4 with H3. HMBC spectra showed long-range coupling C8a (δ 157.9 ppm) with H8, H5, and H4 respectively. C4a (δ 136.4 ppm) showed long range coupling with H3 and H6 and also with H5 and H8. CN (δ 117.2 ppm) showed coupling with H3 and H4.

In DMSO the ^1H NMR peaks for OH-7 and OH-8 and their coupling with H7 and H8 was also observed. Measurements in both solvents are reported in the experimental section of the thesis. Based on the correlations it was confirmed that compound had had a 7*S*, 8*R* configuration and identified as *cis*-(7*S*,8*R*)-7,8-dihydroxy-5,6,7,8-tetrahydroquinoline-2-carbonitrile (**21**).

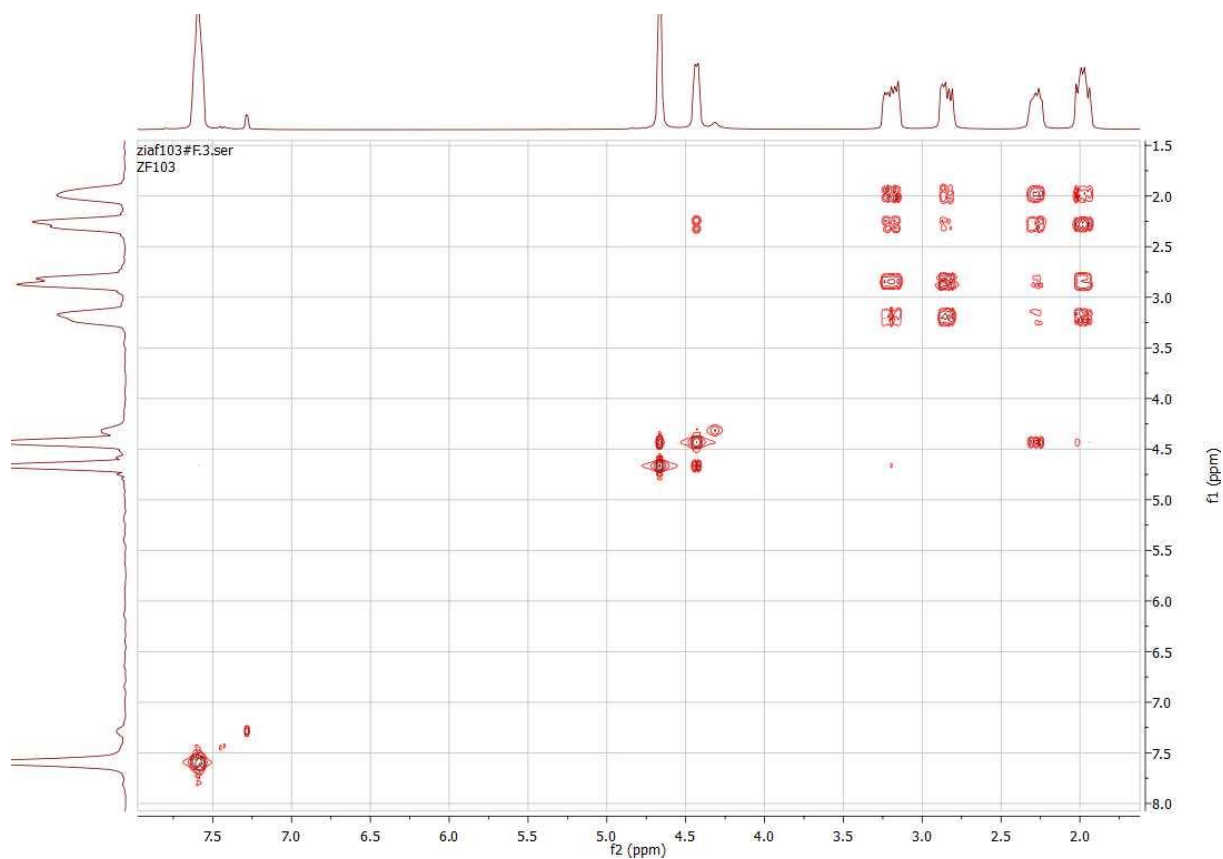


Fig. 4-17 H,H-Cosy spectral data for diol **21**

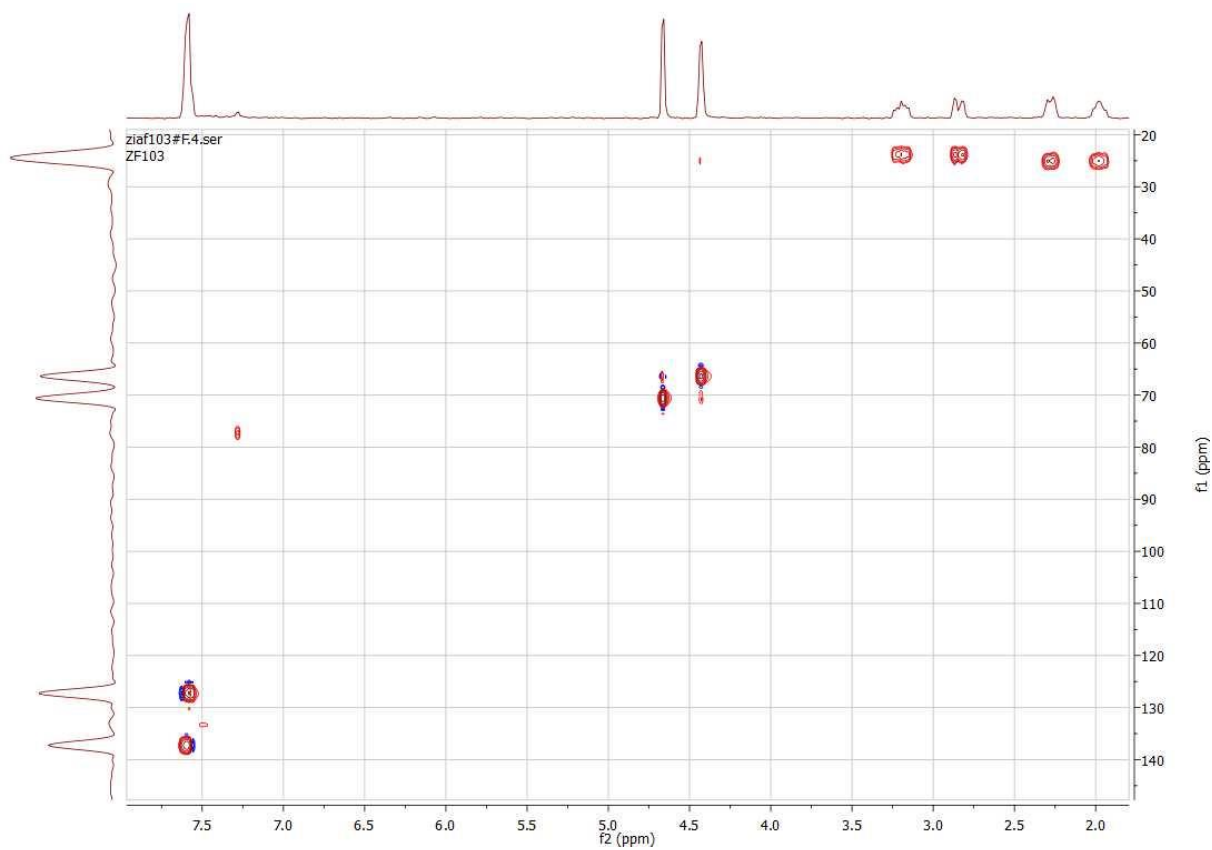
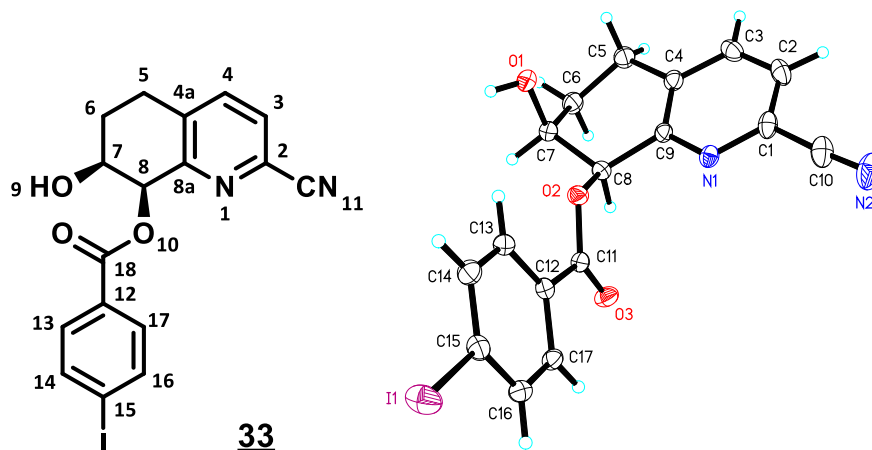


Fig. 4-18 HSQC spectral data for diol **21**

4.7.6 Structure assignment of monoester **33**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Starting from corresponding tetrahydrodiol **21** the structure of monoester **33** was scrutinized. Along with the existing set of peaks for tetrahydrodiol **21** the additional peaks in the aromatic region due to aryl moiety were expected. Also the shift in the signals for 8 was expected due to esterification.

Based on ^1H - ^{13}C correlations it was found that CH_2 -6 (δ 2.10, 2.25 ppm) was directly attached to C6 (δ 24.9 ppm), CH_2 -5 (δ 2.98, 3.22 ppm) with C5 (δ 25.8 ppm), H7 (δ 4.47 ppm) with C7 (δ 67.7 ppm), H8 (δ 6.28 ppm) with C8 (δ 73.1 ppm), H3 (δ 7.56 ppm) with C3 (δ 127.8 ppm), H4 (δ 7.63 ppm) with C4 (δ 138.0 ppm). Aryl-CH 13 and 17 (δ 7.77 ppm) were directly attached to C13 and C17 respectively (δ 131.6 ppm). Aryl-CH 14 and 16 (δ 7.81 ppm) were directly attached to C14 and 16 respectively (δ 137.6 ppm).

^1H - ^1H correlation confirmed vicinal spin network of H6 with H5 and H7, H5 with H6 and H4, H7 with H8, and H6. Aryl CH being away from the ring did not show any coupling with H3, H4, H5, H6, H7, or H8. Aryl-C-I 15 was observed at δ 101.5 ppm and CN 11 was observed at δ 117.3 ppm. Based on the long range coupling and shifts the C4a was observed at δ 137.4 ppm and C8a at δ 154.3 ppm. Aryl carbonate 18 was observed at δ 166.2 ppm. C2 showed long range coupling with H3 and H4 and was observed at δ 129.3 ppm.

The crystal structure analysis also showed that the sample was enantiopure and had a 7S, 8R configuration and identified as *cis*-(7S,8R)-2-cyano-7-hydroxy-5,6,7,8-tetrahydroquinolin-8-yl 4-iodobenzoate (**33**).

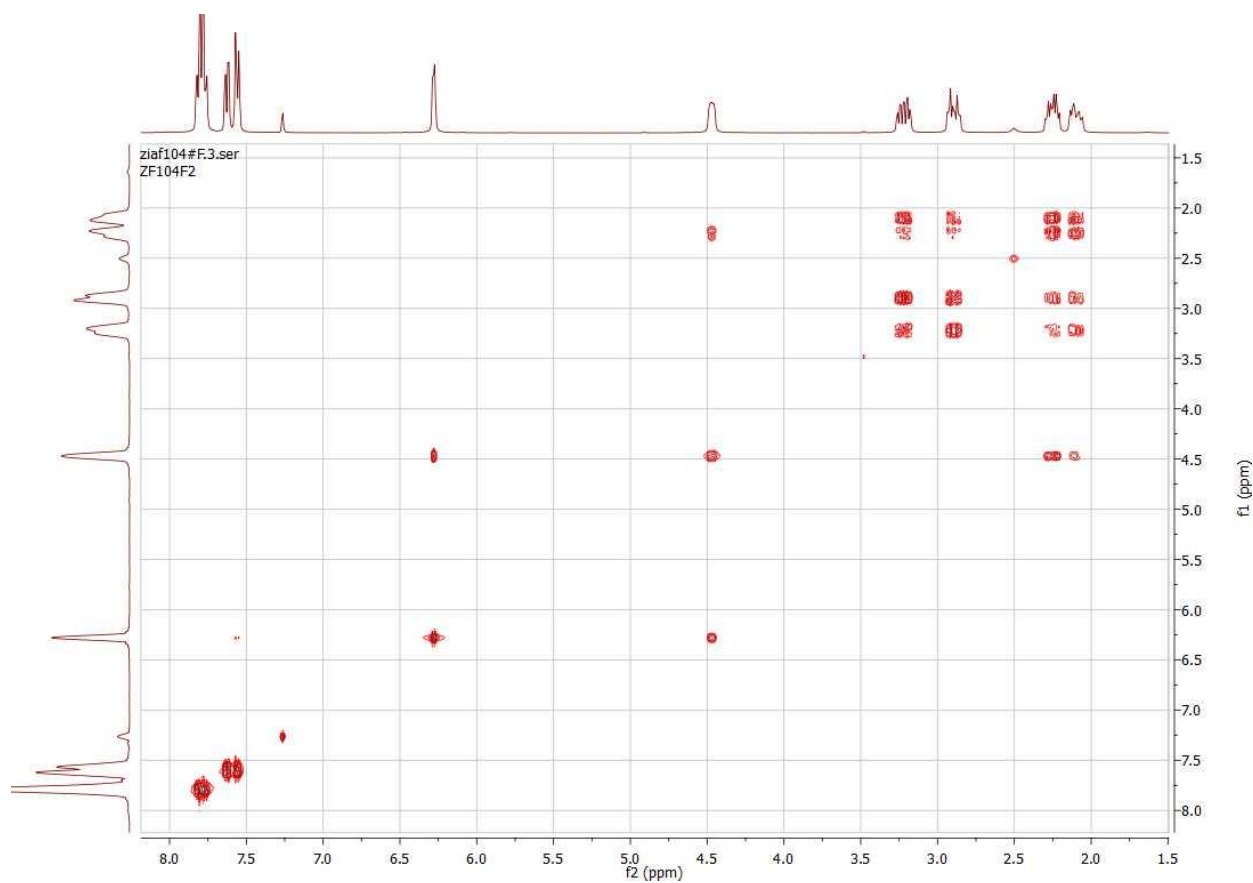


Fig. 4-19 H,H-Cosy spectral data for monoester **33**

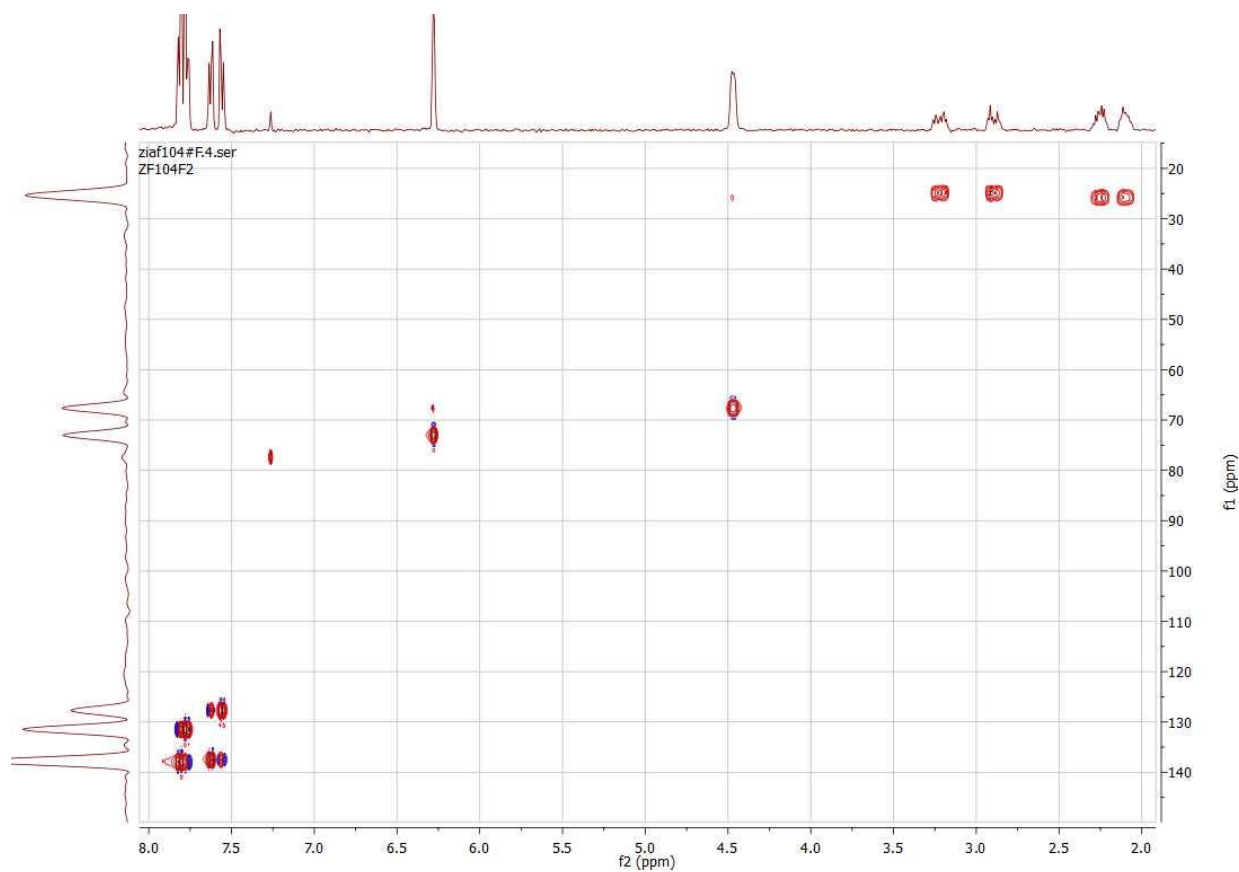
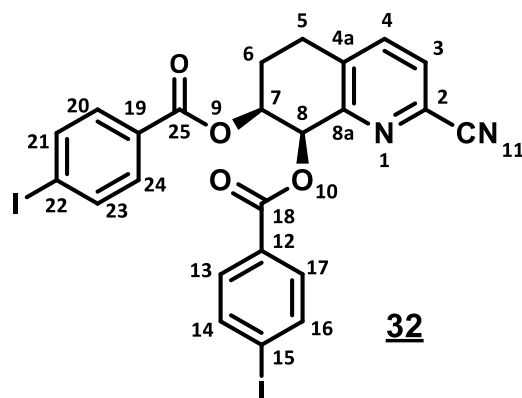


Fig. 4-20 HSQC spectral data for monoester **33**

4.7.7 Structure assignment of diester **32**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Starting from corresponding tetrahydrodiol **21** the structure of diester **32** was scrutinized. Along with the existing set of peaks for tetrahydrodiol **21** the additional peaks in the aromatic region due to aryl moiety were expected. Also the shift in the signals for 7 and 8 was expected due to diesterification.

Based on ^1H - ^{13}C correlations it was found that CH_2 -6 (δ 2.29, 2.46 ppm) was directly attached to C6 (δ 23.8 ppm), CH_2 -5 (δ 3.07, 3.22 ppm) with C5 (δ 25.6 ppm), H7 (δ 5.73 ppm) with C7 (δ 70.2 ppm), H8 (δ 6.54 ppm) with C8 (δ 70.3 ppm), H3 (δ 7.63 ppm) with C3 (δ 128.1 ppm), H4 (δ 7.73 ppm) with C4 (δ 137.9 ppm). Aryl-CH 13 and 17 (δ 7.68 ppm) were directly attached to C13 and C17 respectively (δ 131.6 ppm). Aryl-CH 20 and 24 (δ 7.56 ppm) were directly attached to C20 and C24 respectively (δ 131.5 ppm). Aryl-CH 14 and 16 (δ 7.78 ppm) were directly attached to C14 and C16 respectively (δ 138.0 ppm). Aryl-CH 21 and 23 (δ 7.75 ppm) were directly attached to C21 and C23 respectively (δ 137.9 ppm). Corresponding signals for 11 (δ 117.1 ppm), 15 (δ 101.4 ppm), and 22 (δ 104.5 ppm) were established on the basis of multiplicity and predicted ^{13}C shifts.

^1H - ^1H correlation confirmed vicinal spin network of H6 with H5 and H7, H5 with H6 and H4, H7 with H8, and H6. Aryl CH for both 4-Iodobenzoyl moieties being away from the ring did not show any coupling with H3, H4, H5, H6, H7, or H8. Based on the long range coupling and shifts the C4a was observed at δ 136.7 ppm and C8a at δ 153.9 ppm. Aryl carbonate 18 and 25 were observed at δ 165.3 ppm. C2 showed long range coupling with H3 and H4 and was observed at δ 131.2 ppm.

On the basis of spectral correlation it was assigned that diester had 7S,8R configuration and identified as *cis*-(7S,8R)-2-Cyano-5,6,7,8-tetrahydroquinoline-7,8-diyl bis(4-iodobenzoate) (**32**).

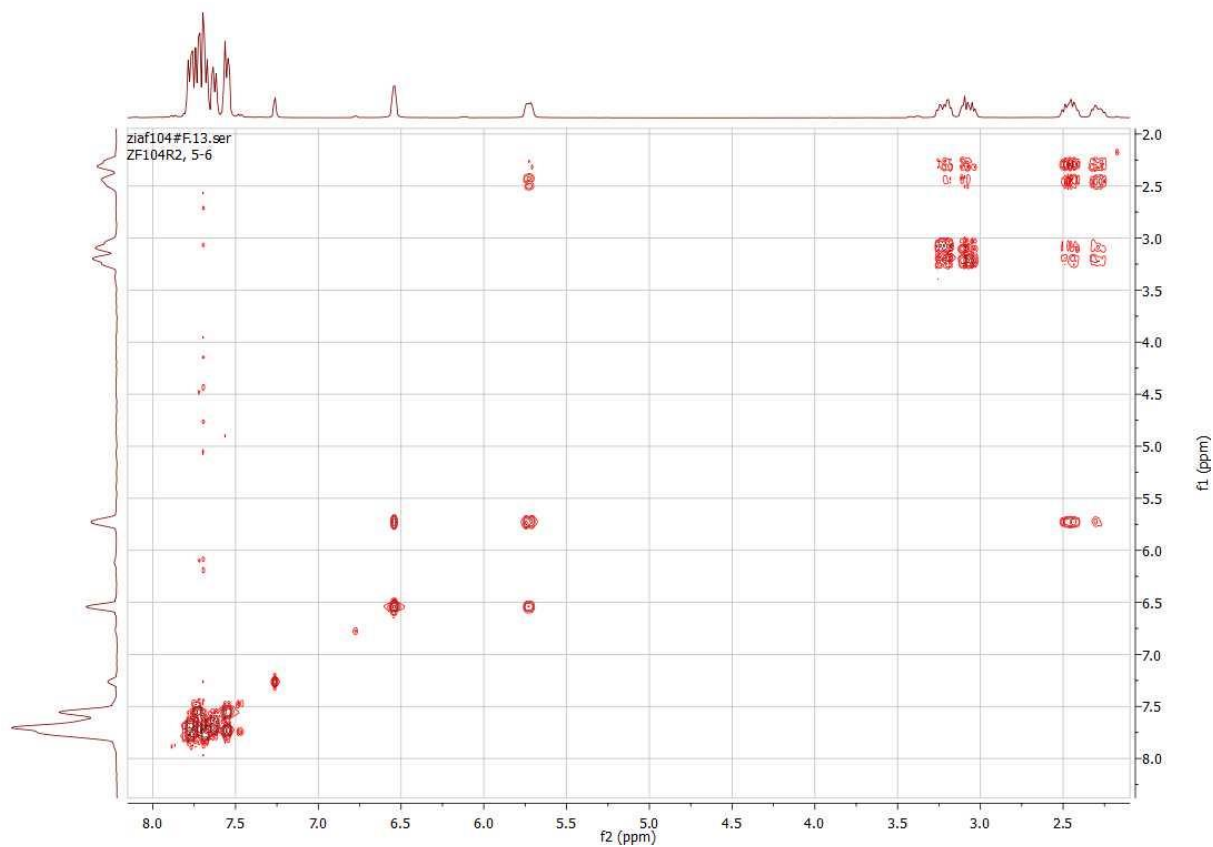


Fig. 4-21 H,H-Cosy spectral data for diester **32**

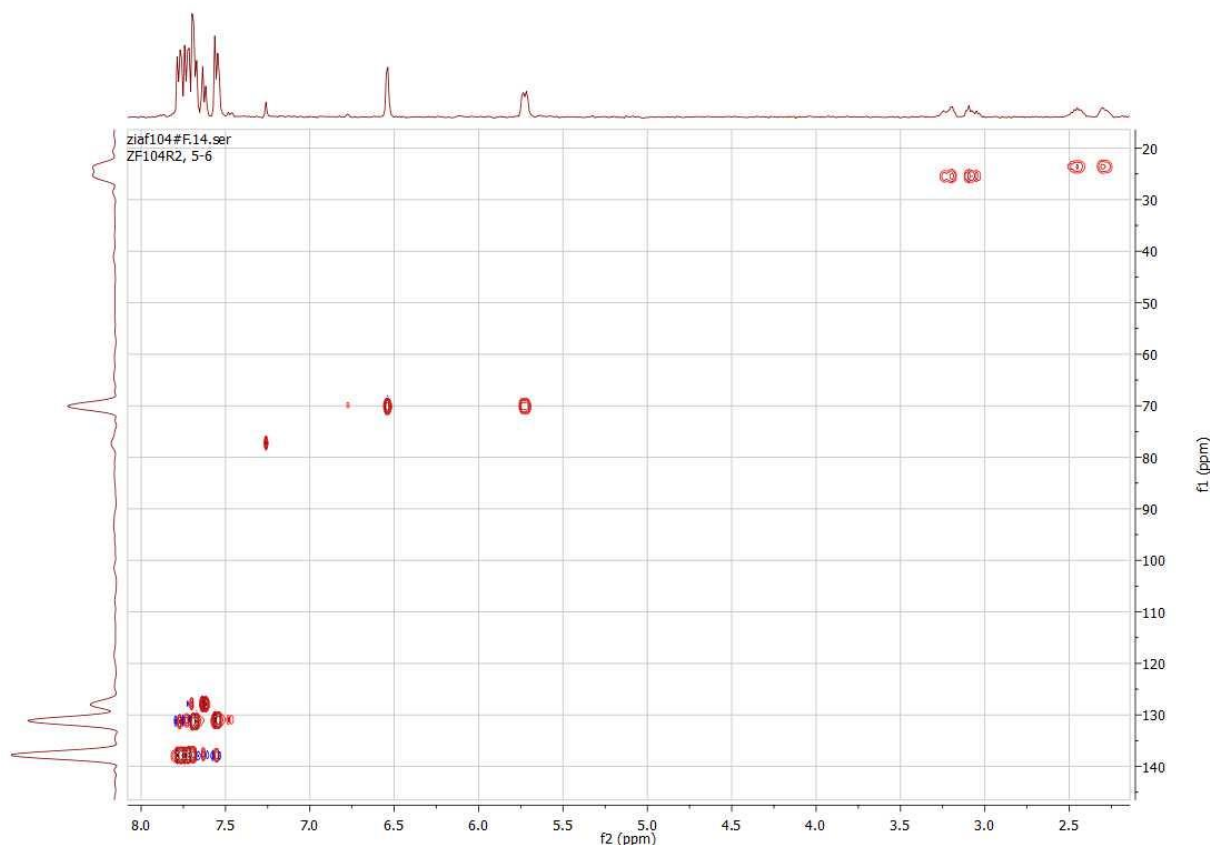
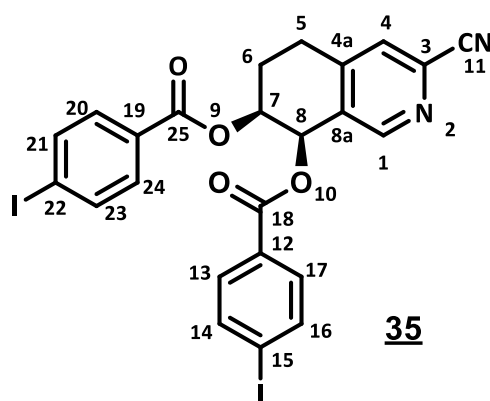


Fig. 4-22 HSQC spectral data for monoester **33**

4.7.8 Structure assignment of diester **35**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Based on ^1H - ^{13}C correlations it was found that CH_2 -6 (δ 2.30, 2.45 ppm) was directly attached to C6 (δ 23.3 ppm), CH_2 -5 (δ 3.10, 3.25 ppm) with C5 (δ 26.0 ppm), H7 (δ 5.68 ppm) with C7 (δ 69.3 ppm), H8 (δ 6.53 ppm) with C8 (δ 67.8 ppm), H1 (δ 8.70 ppm) with C1 (δ 152.3 ppm), and H4 (δ 7.58 ppm) with C4 (δ 128.3 ppm).

Aryl-CH 13 and 17 (δ 7.67 ppm) were directly attached to C13 and C17 respectively (δ 131.3 ppm). Aryl-CH 20 and 24 (δ 7.53 ppm) were directly attached to C20 and C24 respectively (δ 131.1 ppm). Aryl-CH 14 and 16 (δ 7.80 ppm) were directly attached to C14 and C16 respectively (δ 138.1 ppm). Aryl-CH 21 and 23 (δ 7.74 ppm) were directly attached to C21 and C23 respectively (δ 137.8 ppm). Corresponding signals for 11 (δ 117.1 ppm), 15 (δ 101.7 ppm), and 22 (δ 102.0 ppm) were established on the basis of multiplicity and predicted ^{13}C shifts.

^1H - ^1H correlation confirmed vicinal spin network of H6 with H5 and H7, H5 with H6 and H4, H7 with H8, and H6. Aryl CH for both 4-Iodobenzoyl moieties being away from the ring did not show any coupling with isoquinoline ring. Based on the long range coupling and shifts the C4a was observed at δ 132.8 ppm and C8a at δ 147.4 ppm. Aryl carbonate 18 and 25 were observed at δ 165.3 ppm and δ 165.4 ppm. C3 showed long range coupling with H1 and H4 and was observed at δ 129.0 ppm.

On the basis of spectral correlation it was assigned that diester had 7S,8R configuration and identified as *cis*-(7S,8R)-3-cyano-5,6,7,8-tetrahydroisoquinoline-7,8-diyl bis(4-iodobenzoate) (**35**).

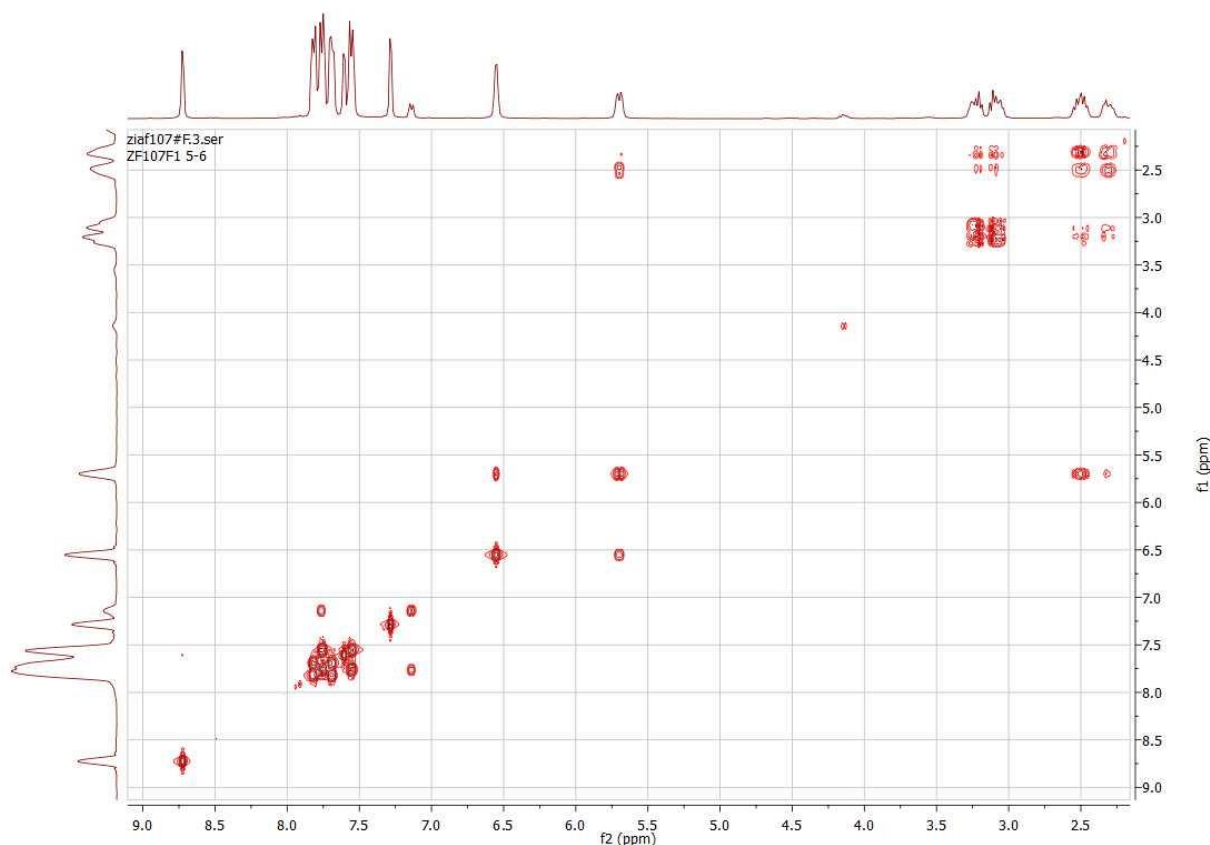


Fig. 4-23 H,H-Cosy spectral data for diester **35**

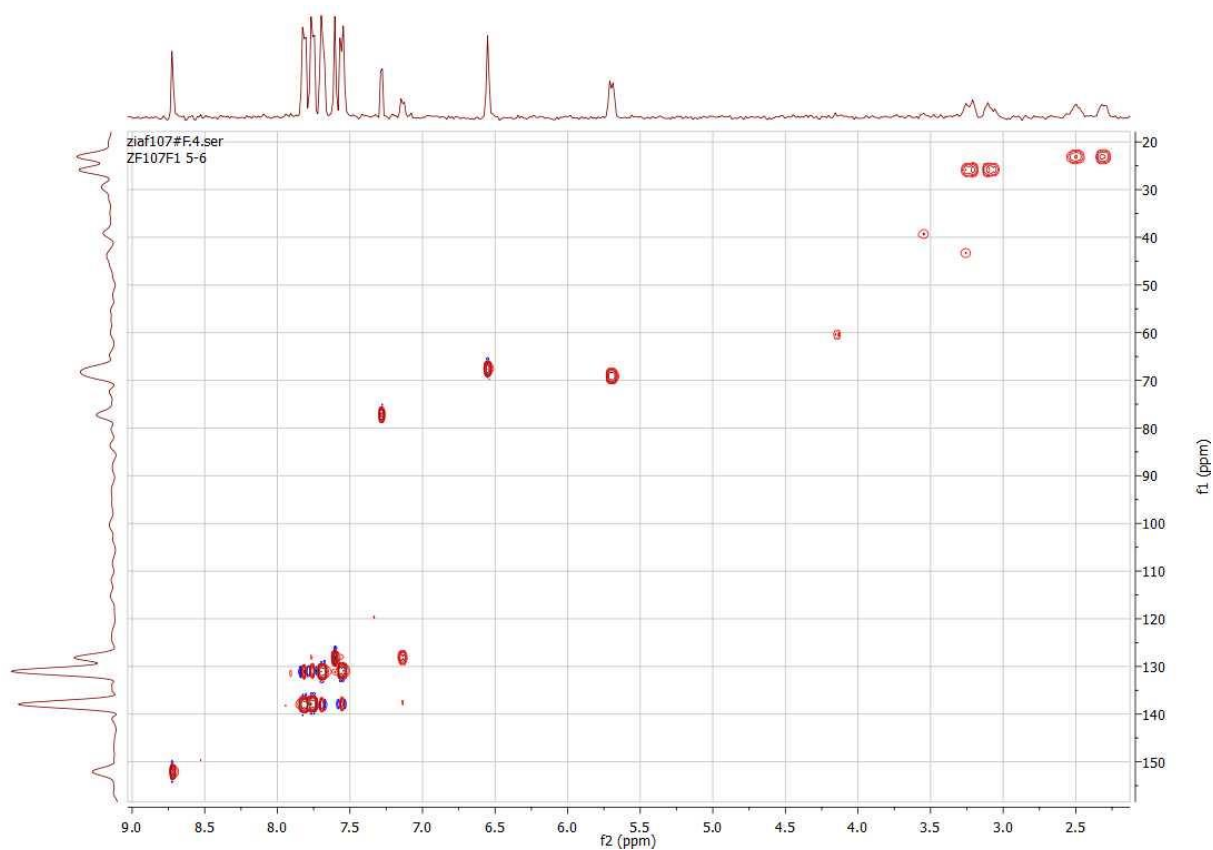


Fig. 4-24 HSQC spectral data for monoester **35**

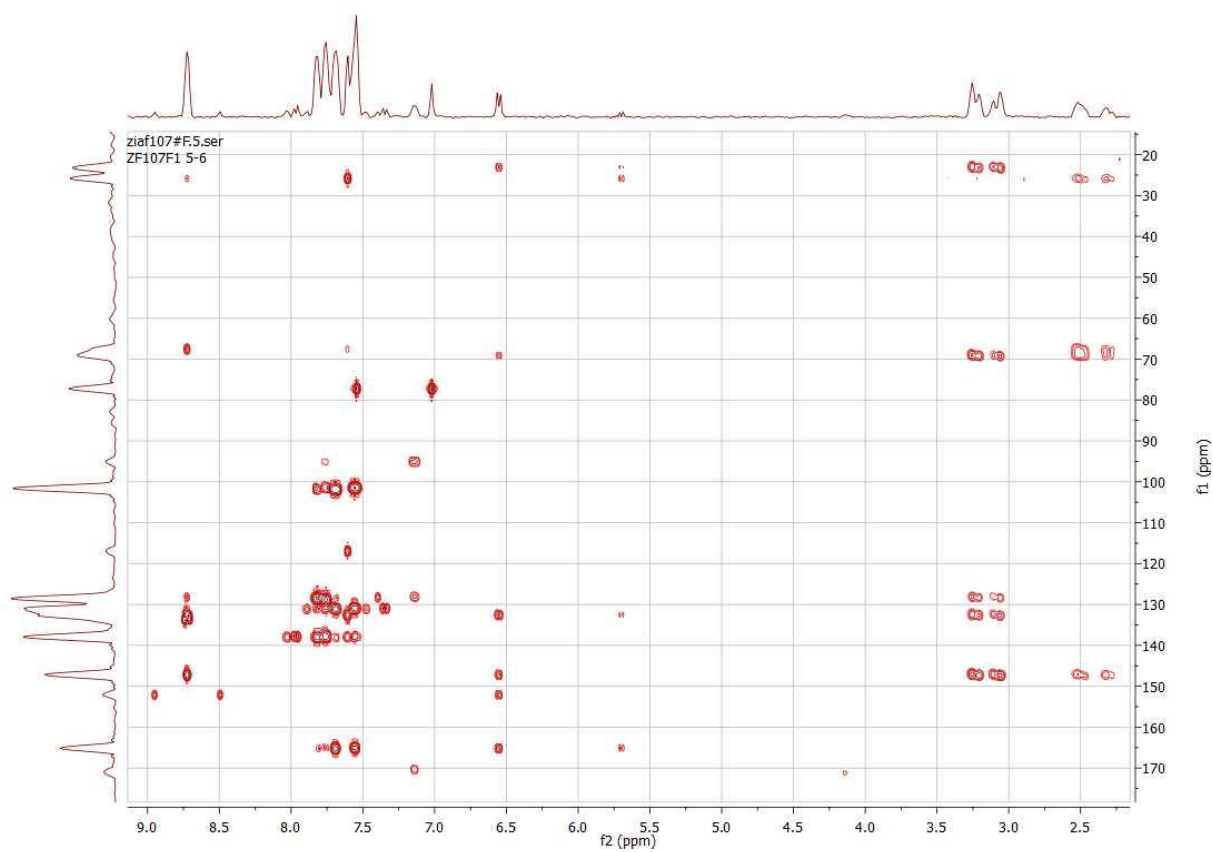
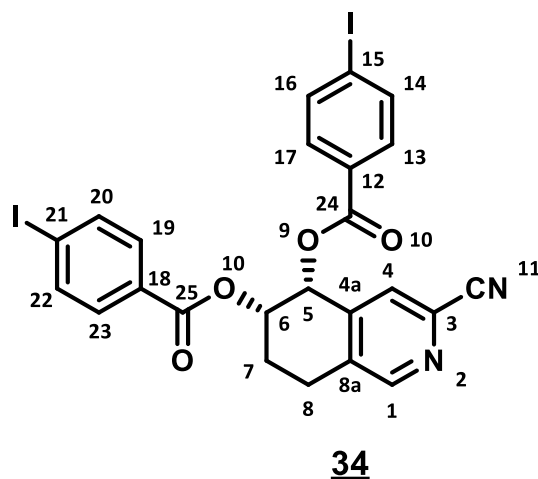


Fig. 4-25 HMBC spectral data for monoester **35**

4.7.9 Structure assignment of diester **34**

Peak assignment was performed based on ^1H -, ^{13}C - and APT-spectra together with 2-dimensional H,H-Cosy, HSQC-, HMBC-, and NOESY- experiments.



Based on ^1H - ^{13}C correlations it was found that CH_2 -7 (δ 2.33, 2.53 ppm) was directly attached to C7 (δ 24.1 ppm), CH_2 -8 (δ 3.10, 3.24 ppm) with C8 (δ 23.2 ppm), H6 (δ 5.81 ppm) with C6 (δ 68.9 ppm), H5 (δ 6.39 ppm) with C5 (δ 68.6 ppm), H4 (δ 7.66 ppm) with C4 (δ 127.6 ppm), and H1 (δ 8.65 ppm) with C1 (δ 151.9 ppm). Aryl-CH 19 and 23 (δ 7.53 ppm) were directly attached to C19 and C23 respectively (δ 131.1 ppm). Aryl-CH 13 and 17 (δ 7.69 ppm) were directly attached to C13 and C17 respectively (δ 131.4 ppm). Aryl-CH 20 and 22 (δ 7.76 ppm) were directly attached to C20 and C22 respectively (δ 138.1 ppm). Aryl-CH 14 and 16 (δ 7.83 ppm) were directly attached to C14 and C16 respectively (δ 138.3 ppm). Corresponding signals for 11 (δ 117.2 ppm), 15 (δ 101.7 ppm), and 21 (δ 102.2 ppm) were established on the basis of multiplicity and predicted ^{13}C shifts.

^1H - ^1H correlation confirmed vicinal spin network of H7 with H8 and H6, H8 with H7, H5 and H1, H6 with H7, and H5, H5 with H6 and H8. Aryl CH for both 4-Iodobenzoyl moieties being away from the ring did not show any coupling with isoquinoline ring. Based on the long range coupling and shifts the C4a was observed at δ 142.9 ppm and C8a at δ 136.3 ppm. Aryl carbonate 24 and 25 were observed at δ 165.3 ppm and δ 165.5 ppm. C3 showed long range coupling with H1 and H4 and was observed at δ 132.2 ppm.

On the basis of spectral correlation it was assigned that diester had 5R, 6S configuration and identified as *cis*-(5R,6S)-3-Cyano-5,6,7,8-tetrahydroisoquinoline-5,6-diyl bis(4-iodobenzoate) (**34**).

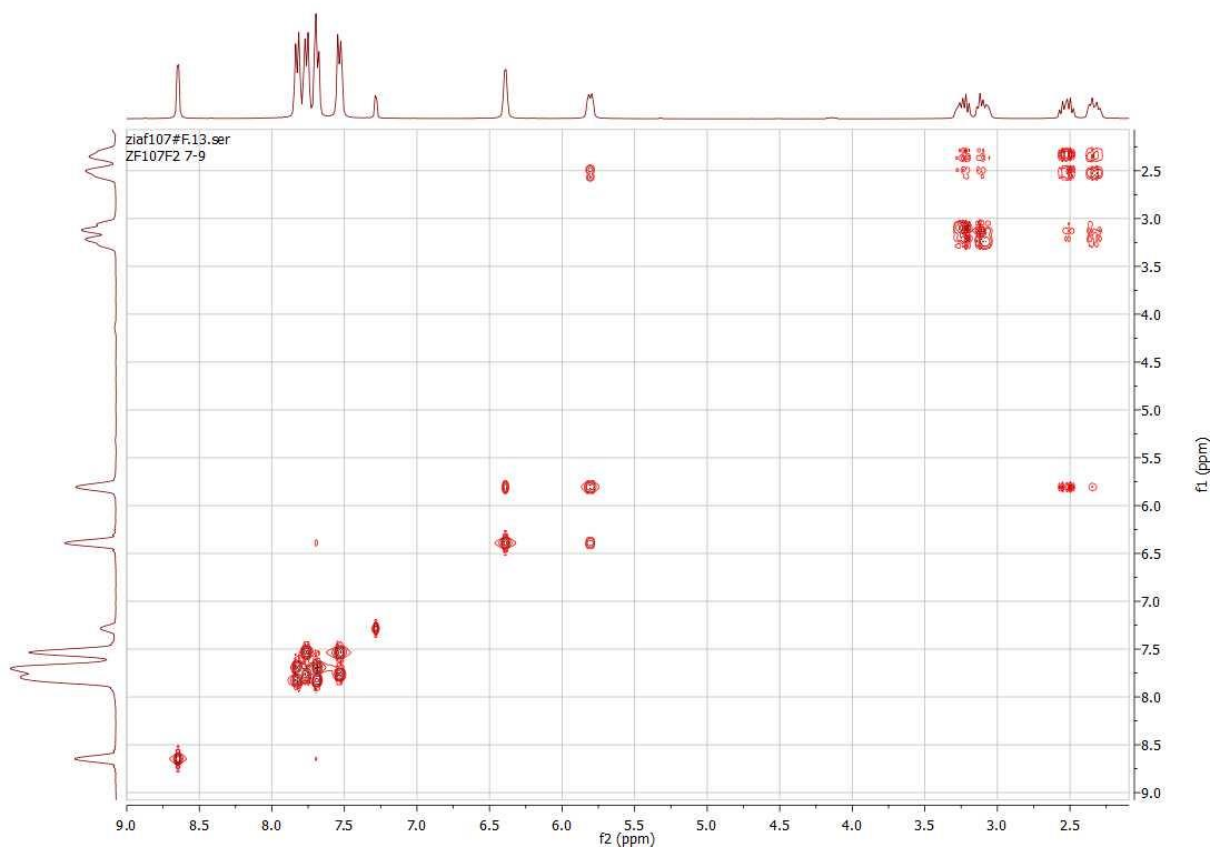


Fig. 4-26 H,H-Cosy spectral data for diester **34**

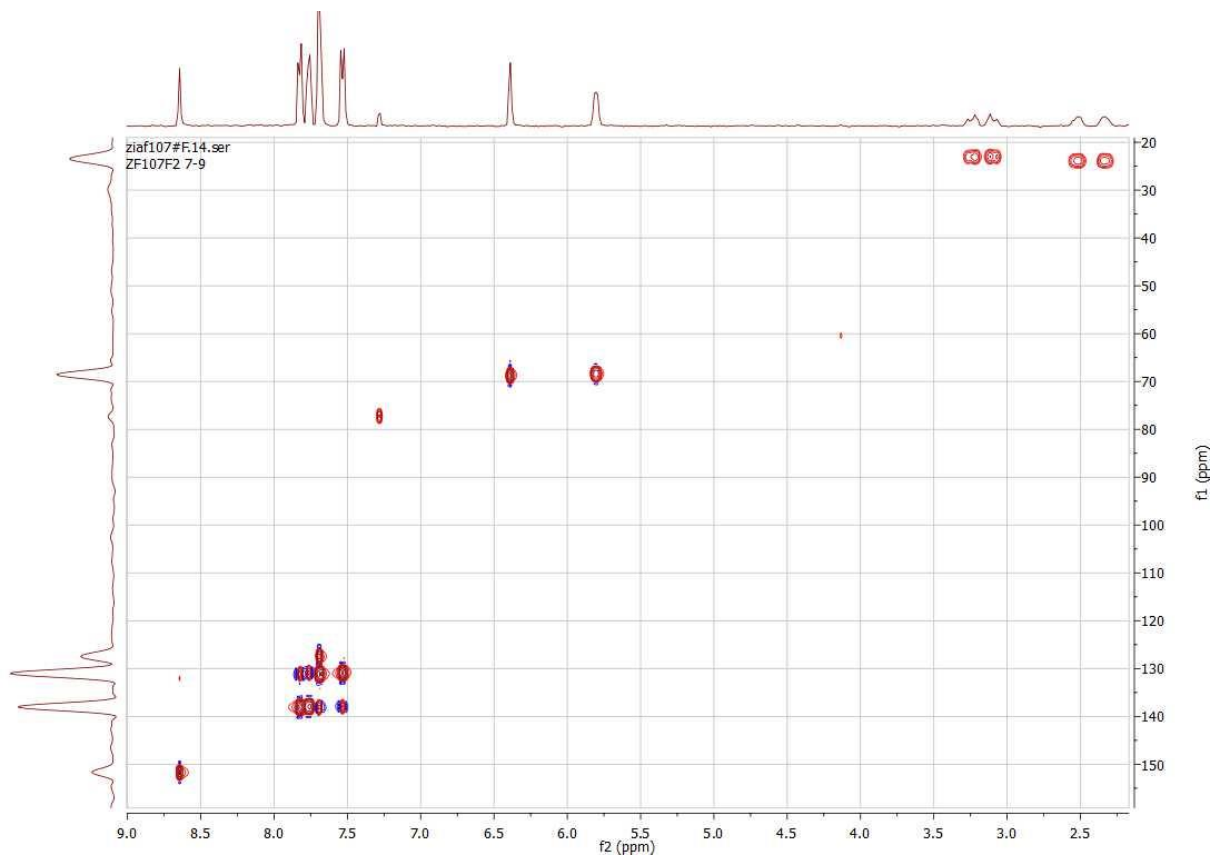


Fig. 4-27 HSQC spectral data for monoester **34**

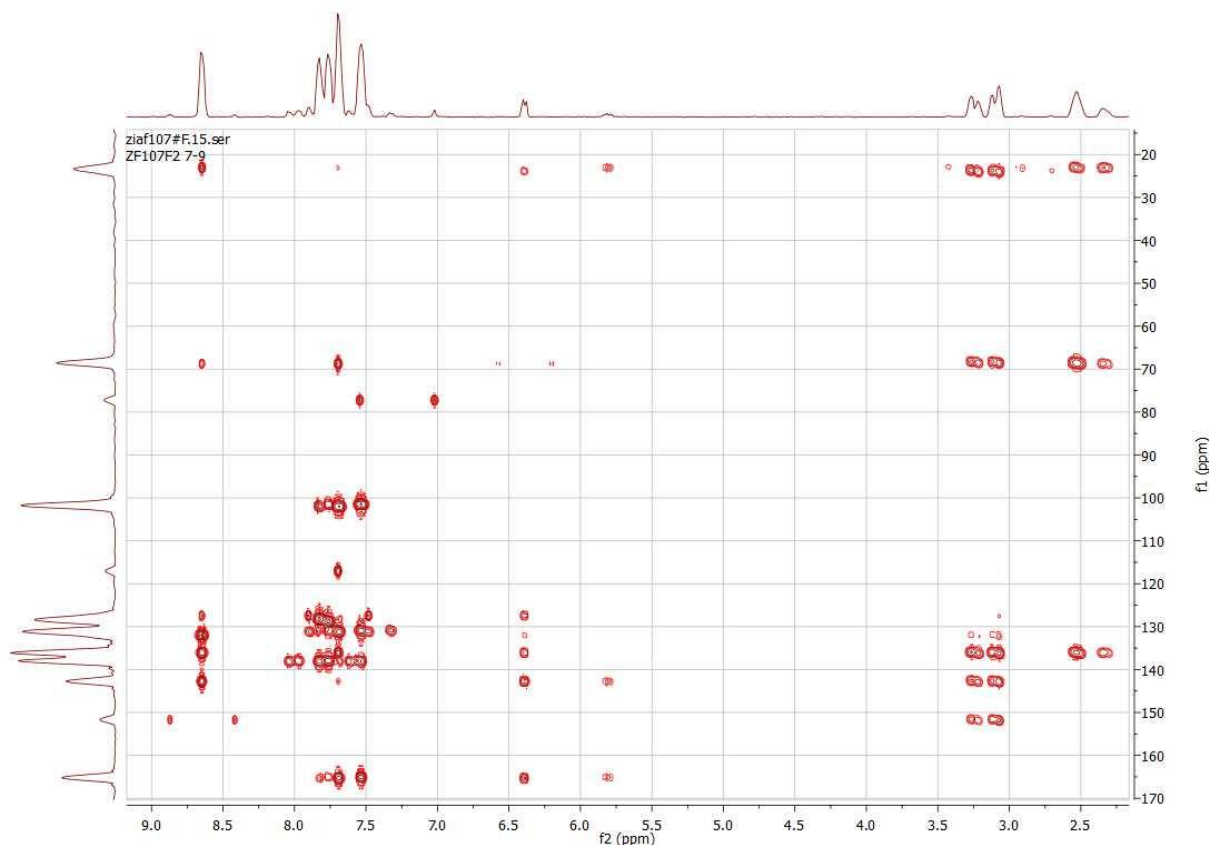
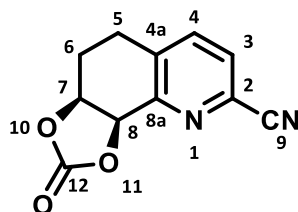


Fig. 4-28 HMBC spectral data for monoester **34**

4.7.10 Structure assignment of cyclic carbonate **39**



39

Starting from corresponding tetrahydrodiol **21**, structure of cyclic carbonate **39** was assigned. Cyclic carbonate **39** had same sets of signals for the ^1H NMR spectra as for tetrahydrodiol **21** with the additional carbonyl signal in the ^{13}C NMR.

Based on ^1H - ^{13}C correlations it was found that CH_2 -6 (δ 1.89-2.30 ppm) was directly attached to C6 (δ 22.5 ppm), CH_2 -5 (δ 2.77- 3.03 ppm) with C5 (δ 25.1 ppm), H7 (δ 5.41 ppm) with C7 (δ 75.4 ppm), H8 (δ 5.82 ppm) was directly attached to C8 (δ 75.1 ppm), H3 (δ 7.99 ppm) with C3 (δ 129.0 ppm), H4 (δ 8.05 ppm) with C4 (δ 138.8 ppm). C4a and C8a were observed at δ 139.1 and 151.7 ppm whereas CN was observed at δ 117.1 ppm. Carbonyl CO was observed at δ 154.0 ppm.

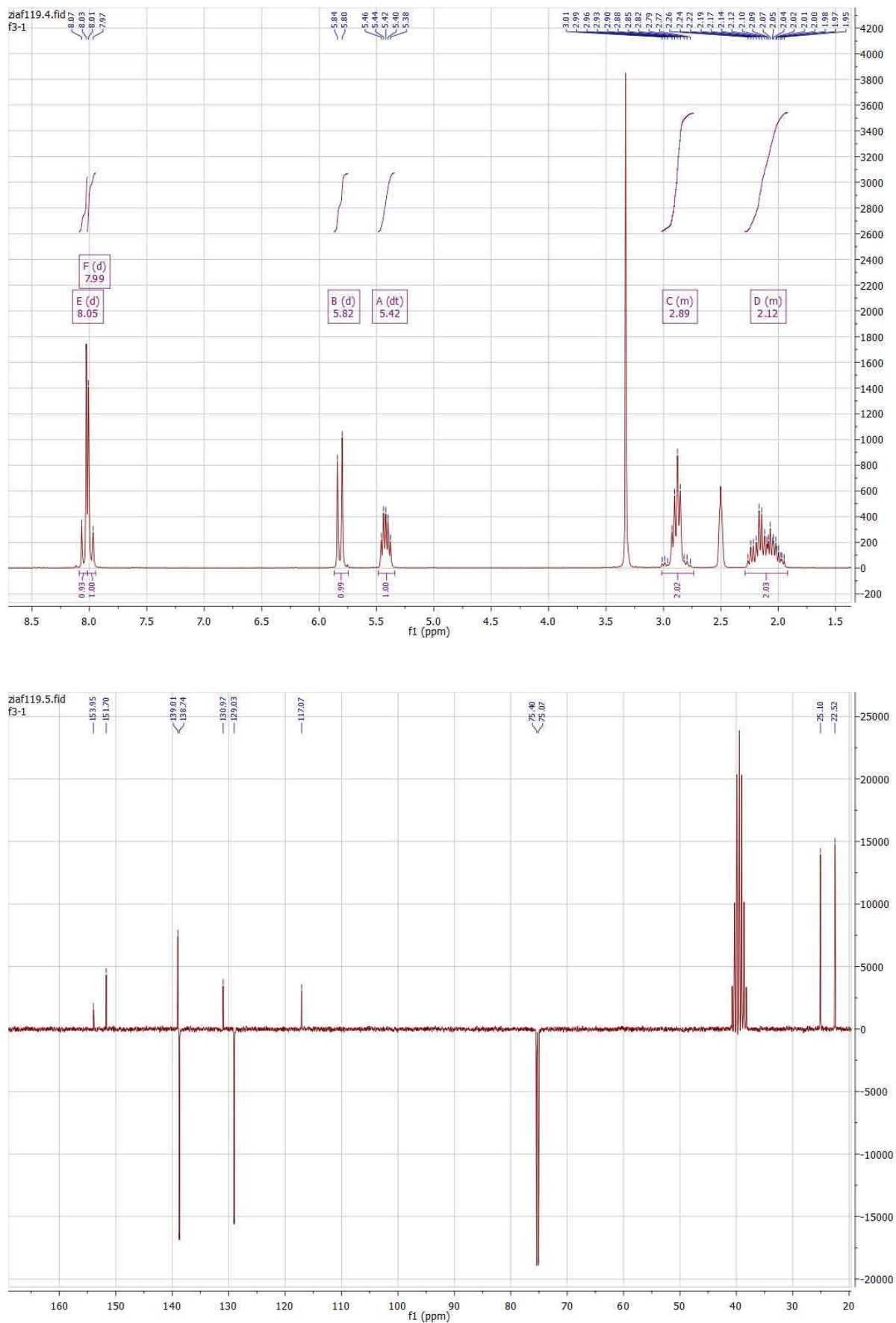


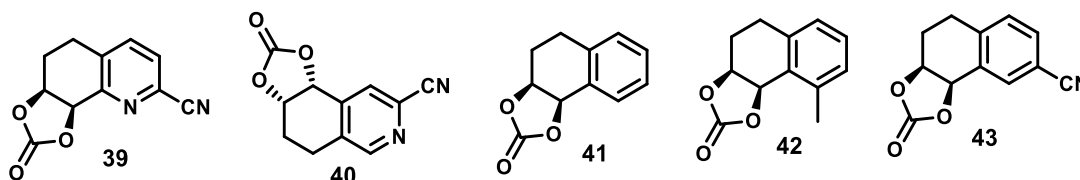
Fig. 4-29 Spectral data for cyclic carbonate **39**

4.8 Elaboration of cyclic carbonates synthesis

4.8.1 Cyclic carbonates of diols

Tetrahydro diol **21** of quinoline-2-carbonitrile **10** was reacted with trichloroacetyl chloride in order to synthesize trichloroacetate. It was expected to form corresponding mono- and di-esters of trichloroacetyl chloride but single product was isolated being the cyclic carbonate **39**. When searched for literature precedence for cyclic carbonate formation by reaction of *cis*-diols with trichloroacetyl chloride, no examples were found. It was found that trichloromethyl chloroformate,²⁰⁴ carbon monoxide and phosgene were the reagents used for protection of *cis*-diols to form cyclic carbonates. Trichloromethyl chloroformate is very expensive reagent for the carbonate formation while trichloroacetyl chloride is many folds cheaper and reaction yields are also comparable. Bestetti *et al* used *cis*-tetrahydronaphthalene-1,2-diol and reacted it with triphosgene in DCM using pyridine as base to synthesize the cyclic carbonate **41**.²⁰⁵

After the formation of cyclic carbonate was observed by reaction with trichloroacetyl chloride, it was tried on number of different diols to study the scope of carbonate formation with trichloroacetyl chloride.



4.8.2 Carbonate reagent comparison

Cyclic carbonate formation was compared by using different reagents. Trichloromethyl chloroformate gave quantitative yields of cyclic carbonate. Trichloroacetyl chloride afforded cyclic carbonate in very good yield. Freshly distilled trichloroacetyl chloride produced 84% cyclic carbonate while technical grade used from shelf gave 81% of cyclic carbonate. There was no major difference in yields when freshly distilled or technical grade trichloroacetyl chloride was used. Table 4-10 shows the comparative yields and the products formed were identical which was verified by NMR and melting point.

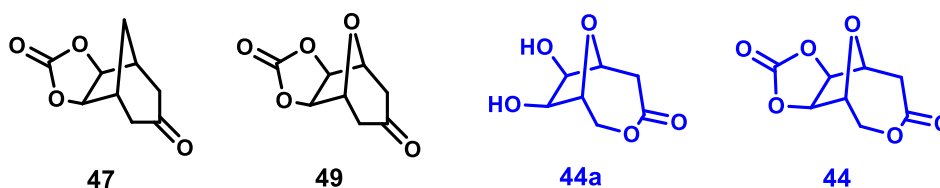
²⁰⁴ [204] R. J. Linderman, N. S. Cutshall and B. T. Becicka, *Tetrahedron Lett.* **1994**, 35, 6639-6642.

²⁰⁵ [205] F. Orsini, G. Sello and G. Bestetti, *Tetrahedron: Asymmetry* **2001**, 12, 2961-2969.

Table 4-10 Comparison of carbonate ester 39 with different reagents

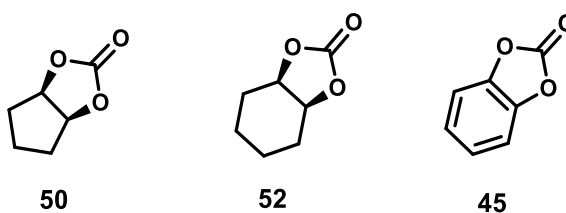
Reagent	Carbonate Yield	Melting Point
Trichloromethyl chloroformate	96%	239-242 °C
Trichloroacetyl chloride (technical)	81%	239-242 °C
Trichloroacetyl chloride (freshly distilled)	84%	239-242 °C

4.8.3 Cyclic carbonates of bicyclic diols



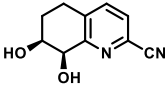
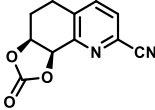
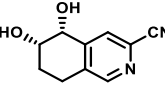
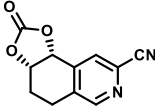
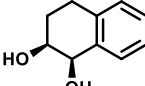
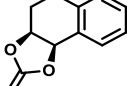
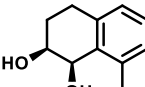
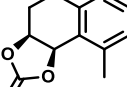
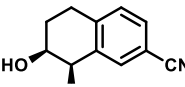
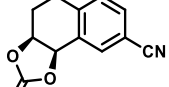
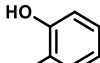
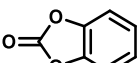
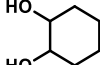
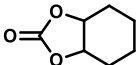
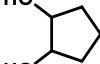
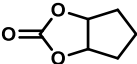
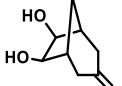
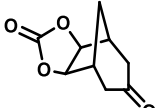
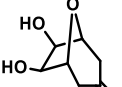
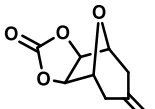

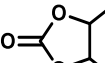

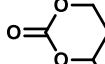
In order to synthesize the cyclic carbonate **44**, oxo diol **44a** (18 mg, 0.103 mmol) dissolved in 3 mL of dry DCM was reacted with trichloroacetyl chloride (41.35 mg, 0.227 mmol, 2.2 eq) in presence of triethylamine (34.4 mg, 0.340 mmol, 3.3 eq). The diol **44a** decomposed TLC showing multiple spots hence no carbonate formation was observed. Diols **46** and **48**, however, gave corresponding cyclic carbonates **47** and **49** as the only products with moderate to high yields.

4.8.4 Cyclic carbonates of cyclic diols

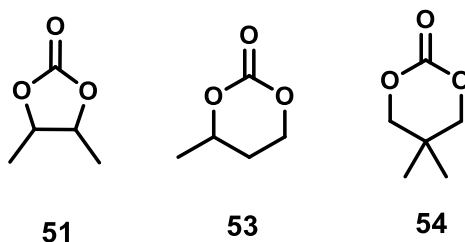


In order to investigate the scope of trichloroacetyl chloride towards the synthesis of cyclic carbonates, three substrates cyclopentane-1,2-diol **50a**, cyclohexane-1,2-diol **52a**, and pyrocatechol **45a**, were selected. Although the cyclic carbonates were formed, the other products like mono and diesters were also observed and cyclic carbonate was not the only product of the reaction. The carbonate obtained with the pyrocatechol was not stable and at room temperature it generally decomposed to a brownish fluffy material with multiple NMR signals. Cyclic carbonates formed by different diols are summarized in table 4-11.

Table 4-11 Carbonates synthesis with trichloroacetyl chloride

Entry	Diol	Nr.	Carbonate	Nr.	Yield
1		20		39	96
2		25		40	96
3		17		41	83
4		18		42	76
5		20		43	82
6		45a		45	47
7		52a		52	52
8		50a		50	34
9		46		47	75
10		48		49	81
11		51a		51	36
12		53a		53	33

4.8.5 Cyclic carbonates of aliphatic diols



The aliphatic diols gave both cyclic carbonates and diesters when reacted with trichloroacetyl chloride. The diester was the major product of reaction and carbonate was minor product in the selected examples. This indicates that aromatic *cis*-diols can be converted to cyclic carbonates with trichloroacetyl chloride whereas the aliphatic diols are not completely converted to cyclic carbonates.

4.8.6 Scope of cyclic carbonate synthesis with trichloroacetyl chloride

Along with other existing reagents for cyclic carbonate synthesis are CO, phosgene, diphosgene (trichloroacetyl chloroformate), and triphosgene etc. We discovered the synthesis of cyclic carbonate by the use of trichloro acetyl chloride. It can be used efficiently for 1,2-diols with *cis*-configuration. In order to elaborate the scope and limitation of newly discovered method, we chose 12 selected examples for which summarized results are given in the table 4-11. The stability of cyclic carbonates formed varied from very stable in case of quinoline and isoquinoline diol carbonates to highly unstable in case of catechol and aliphatic diol carbonates.

The side products formed especially in case of aliphatic diols were mono- and diesters of corresponding hydroxyl group and sometimes it becomes really a challenge to isolate all products of reaction and isolate the cyclic carbonate from mixture. It can be concluded that the method works well for 6-membered cyclic *cis*-diols with high yields to form 5-membered cyclic carbonate.

4.9 Conclusion

The present work can be summarized in three sections: First section describes the screening studies of various enzymes with available dioxygenases. Second section describes the preparative scale biotransformations in order to isolate and identify the metabolites formed by biotransformations. Third section deals with different methods and strategies applied towards the proof of absolute configuration of isolated metabolites.

Initial screenings were carried out by whole cell mediated biotransformations using four different dioxygenases. The dioxygenases used were *Ralstonia eutropha* B9 expressing BZDO, *E. coli* JM109 (pDTG601A) expressing TDO, *E. coli* JM109 (DE3) (pDTG141) expressing NDO (NDO1), and *E. coli* JM109 (PVL1343-PMS13) expressing NDO (NDO2). Screening scale biotransformations with BZDO were not successful and therefore, no preparative scale biotransformations were performed with BZDO.

Regioselective *cis*-dihydroxylation of the carbocyclic ring in the naphthalenes, quinolines and isoquinolines at 5,6 and/or 7,8 bonds occurred to give the corresponding *cis*-dihydrodiol metabolites. Regioselective *cis*-dihydroxylation of the heterocyclic rings in the quinolines and isoquinolines at 2,3 and/or 3,4 bonds could not be observed at such small scale reactions.

Five substituted naphthalene substrates (1-methyl- **1b**, 2-methyl- **1c**, 2-bromo- **1d**, 2-cyano- **1e**, and 2-methoxy- **1f**) were subjected to screening with dioxygenases. All substrates were accepted by all three dioxygenases TDO, NDO1, and NDO2. Preparative scale biotransformation of 1-methyl naphthalene **1b**, and 2-methyl naphthalene **1c**, gave corresponding 7,8 diols and also hydroxylation on the Me side chain was observed. 2-Bromo naphthalene **1d** and 2-cyano naphthalene **1e** were also converted to corresponding 7,8 diols. This might be due to solubility problems of the substrate and transfer of the substrate across the cell wall in order to get transformed.

From the quinoline series 10 substrates were subjected to screening with DOs. In case of methyl quinoline-2-carboxylate **2b**, 2-(tribromomethyl)-quinoline **2e**, bisquinoline **2f**, and 3-methylquiniline **2g**, no products were observed in the HPLC analysis and only starting materials were recovered. 3-Bromoquinoline-2-carboxamide **2i** and 3-bromoquinoline-2-carbonitrile **2j** were also not converted by any of the DOs. The products were formed in case of quinoline-2-carboxylic acid **2c**, quinoline-2-carboxamide **2d** as confirmed by HPLC

analysis but due to lower concentrations and on small scale it was not possible to isolate the products from the media due to higher solubility of diols formed. Quinoline-2-carbonitrile **2a** gave corresponding 7,8 diol and 3-bromoquinoline **2h** gave mixture of 5,6 and 7,8 diols with 7,8 diol being the minor product formed in traces.

From the isoquinoline series 8 substrates were screened with DOs and isoquinoline-1-carbonitrile **3b** and isoquinoline-3-carbonitrile **3d** gave mixture of both 5,6 and 7,8 diols. Few attempts were made to perform the biotransformations at fermenter scale in order to understand the upscale possibility of dioxygenase dihydroxylations.

Due to unstable nature of diols and rearomatization due to elimination of water, it was necessary to reduce the adjacent olefinic bond on the hydrocarbon ring. The reduced diols were relatively more stable than corresponding dihydrodiols. In order to prove the absolute configuration the diols were converted to esters and then the esters were crystallized and subjected to X-ray crystallography. Camphorsulfonate ester from tetrahydro diol of quinoline-2-carbonitrile could not be crystallized. The diols were then reacted with 4-iodobenzoyl chloride in order to synthesize the 4-iodobenzoate esters. Due to the presence of heavy I atom, the X-ray crystallographic study of ester could establish the absolute chemistry of corresponding diol. Diesters formed with 4-iodobenzoyl chloride were not good candidates for crystallization as they behaved like super-cooled glass like material and several attempts to crystallize generally resulted in glass like solid material. The monoesters of 4-iodobenzoyl chloride formed nice crystals that could be used to prove the absolute configuration of diols.

Through the process of derivatization of diols produced by dihydroxylations it was found out that trichloroacetyl chloride can be used as reagent for protection of *cis*-diols as 5-membered cyclic carbonates. The scope and limitations of trichloroacetyl chloride towards 5-membered cyclic carbonate synthesis was elaborated with selected examples from different classes of diols. The aliphatic diols gave both cyclic carbonates and diesters with trichloroacetyl chloride. The diester was the major product of reaction as compared to the carbonate. This indicates that aromatic *cis*-diols can be protected as 5-membered cyclic carbonates with trichloroacetyl chloride whereas the aliphatic diols are not completely converted to cyclic carbonates.

5 Experimental Part

In this chapter results for the experimental work will be provided in detail. The first part will deal with general materials and methods. In the second part preparative scale biotransformation experiments and resulting dihydroxylated diols will be described in detail. In the third part derivatization and experiments for the proof of their absolute configuration will be described. In the next section synthesis of cyclic carbonate esters of diols and boronic esters will be presented. In the last section experiments about relative substrate conversion by different dioxygenases used will be described.

5.1 General

Unless noted otherwise, all reagents and microbial growth media were purchased from commercial suppliers and used without further purification. DCM, Et₂O, dioxane, MeOH, THF and toluene intended for water-free reactions were pre-distilled and then desiccated on Al₂O₃ columns (PURESOLV, Innovative Technology). All chromatography solvents were distilled prior to use.

Flash column chromatography was performed on silica gel 60 from Merck (40-63µm) using LP and Et₂O or EtOAc mixtures. Automated column chromatography was performed on a Büchi Sepacore Flash System (2 x Büchi Pump Module C-605, Büchi Pump Manager C-615, Büchi UV Photometer C-635, Büchi Fraction Collector C-660). Basic silica gel was obtained by mixing Et₃N (5% of solvent volume), silica gel and the desired solvent mixture.

Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected.

NMR-spectra were recorded from CDCl₃, DMSO-d₆, MeOD, or D₂O solutions on a Bruker AC 200 (200 MHz) or Bruker Avance UltraShield 400 (400 MHz) spectrometer and chemical shifts are reported in ppm using tetramethylsilane (TMS) as internal standard. Peak assignment is based on correlation experiments. Ambiguous assignment is marked with an asterisk.

General conversion control and examination of purified products were performed with GC Top 8000 / MS Voyager (quadropole, EI+) or GC Focus / MS DSQ II (quadropole, EI+) using a standard capillary column BGB5 (30mx0.32mm ID).

HPLC analyses were recorded on a Thermo Finnigan Surveyor Plus using a Phenomenex Luna C18 (2) column (10 μ , 250mmx4.6mm diameter). Detection was performed on a Thermo Finnigan PDA Plus photodiode array detector. (Standard method: 1mL/min, H₂O (0.1%TFA)/ACN = 85:15 1min \rightarrow H₂O (0.1%TFA)/ACN = 20:80 9min \rightarrow H₂O (0.1%TFA)/ACN = 20:80 3min \rightarrow H₂O (0.1%TFA)/ACN = 85:15 3min \rightarrow H₂O (0.1%TFA)/ACN = 85:15 5min)

Combustion analyses were carried out in the Microanalytic Laboratory, University of Vienna and reports are included in the thesis. HRMS were recorded using a Shimadzu LC prominence (Phenomenex Luna C 18(2) 5 μ , 300mmx4.6mm diameter) /MS-IT-TOF. The MS was calibrated in ESI mode and measurements were recorded in APCI mode.

Centrifugations for harvesting of cells were realized on bench centrifuge: Eppendorf 5804R centrifuge, Beckmann J2-21M/E centrifuge (JA-10 rotor) or Sigma 6K15 centrifuge (rotor 372/C). Centrifugations for removing of cell debris were realized on Beckmann J2-21M/E centrifuge (JA-17 rotor), Sigma 3K30 (rotor 19777).

Specific rotation value $[\alpha]_D^{20}$ was determined using a Perkin Elmer Polarimeter 241 by the following equation: $[\alpha]_D^{20} = 100 \cdot \alpha / (c \cdot l)$; c[g/100 mL], l[dm]. Later with the availability of modern equipment, specific rotation for some compounds was measured on an Anton Paar MCP500 Polarimeter at the specified conditions.

Dip reagent: 1.0 g KMnO₄
 20 g K₂CO₃
 10 mL of NaOH solution (5%)
 150 mL water

Dip reagent: 13.2 g conc. sulfuric acid
 0.8 g cerium(IV) ammonium nitrate
 10.0 g phosphorus molybdate
 150 mL ethanol

Bacteria on Agar plates were incubated in an Heraeus Instruments FunctionLine incubator under air. Agar plates were prepared with LB medium supplemented by 1.5% w/v

Agar Agar. Bacterial cultures were incubated in baffled Erlenmeyer flasks containing 20% v/v media in orbital shakers (Thermoshake, Gerhardt) and (InforsHT Multitron 2 Standard) at 200 rpm and at specified temperature. All materials and biotransformation media were sterilized by autoclaving at 121 °C for 20 minutes. Various aqueous stock solutions were sterilized by filtration through 0.20 µm syringe filters.

Large scale (2 L) fermentations have been performed in a New Brunswick Bioflow 110 fermenter equipped with pH-probe, oxygen probe, flow controller and temperature control. Monitoring of all fermentation parameters was performed using the Biocommand Plus 3.30 software by New Brunswick.

5.2 Media for biotransformation

5.2.1 Hutner's Mineral Base Medium (HMB)

HMB Medium was prepared according to Myers *et al.*¹²⁷

400 mg	KOH (dissolved in 500 mL dist. Water)
200 mg	Nitrilotriacetic acid
283 mg	MgSO ₄
67 mg	CaCl ₂ x 2 H ₂ O
0.2 mg	(NH ₄) ₂ MoO ₄
2 mg	Fe(II)SO ₄
1 mL	Hutner's Metals 44 solution
1 g	(NH ₄) ₂ SO ₄
2.72 g	KH ₂ PO ₄
7.34 g	Na ₂ HPO ₄ x 10 H ₂ O
dist. H ₂ O	add to 1L

pH adjusted to 6.8 with concentrated HCl

The medium was sterilized by steam sterilization in an autoclave at 121°C for 20 minutes.

5.2.2 Modified Hutner's Mineral Base Medium (HMB): for pH 6.8

200 mg	Nitrilotriacetic acid
283 mg	MgSO ₄
67 mg	CaCl ₂ x 2 H ₂ O
0.2 mg	(NH ₄) ₂ MoO ₄

2 mg	Fe(II)SO ₄
1 mL	Hutner's Metals 44 solution
1 g	(NH ₄) ₂ SO ₄
2.08 g	NaH ₂ PO ₄ x H ₂ O
2.31 g	Na ₂ HPO ₄ x 2 H ₂ O
dist. H ₂ O	add to 1 L

pH adjusted to 6.8 with concentrated HCl

5.2.3 Modified Hutner's Mineral Base Medium (HMB): for pH 7.4

200 mg	Nitrilotriacetic acid
283 mg	MgSO ₄
67 mg	CaCl ₂ x 2 H ₂ O
0.2 mg	(NH ₄) ₂ MoO ₄
2 mg	Fe(II)SO ₄
1 mL	Hutner's Metals 44 solution
1 g	(NH ₄) ₂ SO ₄
872 mg	NaH ₂ PO ₄ x H ₂ O
3.86 g	Na ₂ HPO ₄ x 2 H ₂ O
dist. H ₂ O	add to 1 L

pH adjusted to 7.4 with 2N NaOH

The medium was sterilized by steam sterilization in an autoclave at 121°C for 20 minutes.

5.2.4 Hutner's Metals 44 solution:

Hutner's Metals 44 solution was prepared according to Myers *et al.*¹²⁷

100 µL	conc. H ₂ SO ₄ (dist. water add to 50 mL)
0.5 g	EDTA
2.2 g	ZnSO ₄ x 7 H ₂ O
1 g	Fe(II)SO ₄ x 7 H ₂ O
0.34 g	CuCl
50 mg	Co(II)NO ₃ x 6 H ₂ O
36 mg	Na ₂ B ₄ O ₇ x 10 H ₂ O
dist. water	add to 50 mL

5.2.5 Na/K-buffer (1mmol/L)

Na/K-buffer (1mmol/L) was prepared according to Dorn *et al.*²⁰⁶

140 mg	Na ₂ HPO ₄ x 2 H ₂ O
28 mg	KH ₂ PO ₄
0.1 mg	Ca(NO ₃) ₂ x 4 H ₂ O
0.4 mg	MgSO ₄ x 7 H ₂ O
2 mg	(NH ₄)SO ₄
0.02 mg	Fe(III)NH ₄ -citrat
2 µL	Micronutrient solution SL6
dist. H ₂ O	add to 1L

The medium was sterilized by steam sterilization in an autoclave at 121°C for 20 minutes.

5.2.6 Micronutrient solution SL6: (Pfennig et al.)²⁰⁷

100 mg	ZnSO ₄ x 7 H ₂ O
30 mg	MgSO ₄ X 4 H ₂ O
300 mg	H ₃ BO ₃
200 mg	CoCl ₂ x 6 H ₂ O
10 mg	CuCl ₂ x 2 H ₂ O
20 mg	NiCl ₂ x 6 H ₂ O
30 mg	NaMoO ₄ x 2 H ₂ O
dist. H ₂ O	add to 1 L

5.2.7 LBamp and LBkan

10.0 g	peptone
5 g	yeast extract
10.0 g	sodium chloride
1000 mL	deion. H ₂ O

Sterilized at 121°C for 20 minutes, let it cool down to below 40°C and then added 2 mL ampicillin stock solution to make LBamp and 1.5 mL kanamycin stock solution to make LBkan solution. In order to make LBamp+kan add 4 mL amp and 2 mL kan stock solution.

²⁰⁶ [206] E. Dorn, M. Hellwig, W. Reineke and H. J. Knackmuss, *Archives of Microbiology* **1974**, 99, 61-70.

²⁰⁷ [207] N. Pfennig and K. Lippert, *Archiv für Mikrobiologie* **1966**, 55, 245-256.

5.2.8 Phosphate-buffered saline (10mM PBS)

8.0 g	NaCl (137 mM)
0.2 g	KCl (2.7 mM)
1.44 g	Na ₂ HPO ₄ (10 mM)
0.24 g	KH ₂ PO ₄ (2 mM)
800 mL	deion. H ₂ O

Adjust pH to 7.4 with HCl add water to make total volume of 1L and then Sterilize at 121°C for 20 minutes.

5.2.9 Modified Hutner's Mineral Base Medium (MSB)

MSB medium was prepared by mixing three solutions prepared separately

Solution A: phosphate buffer, pH 7.3, 40 mL L⁻¹

Solution B: Hutner's concentrated base, 10 mL L⁻¹

Solution C: 20% w/v ammonium sulphate, 5 mL L⁻¹

Solution A was prepared by mixing 700 mL of 1M Na₂HPO₄ with 300 mL of 1M KH₂PO₄.

Composition of solution B included following;

20 g	nitrilotriacetic acid (NTA-free acid)
59.3 g	MgSO ₄ .7H ₂ O
6.67 g	CaCl ₂
18.5 mg	(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O
198 mg	FeSO ₄ .7H ₂ O
100 mL	Hutner's Metals 44 solution

To prepare solution B, NTA was dissolved separately in 600 mL water and neutralized with KOH (14.6 g KOH); other constituents were added in given order with constant stirring. Next component was added only after previous component was completely dissolved. pH was adjusted to 6.8 by adding KOH solution drop wise before making to final volume of one liter.

Metals 44 solution was prepared as follows;

In 800 mL distilled water dissolve,

2.5 g	EDTA (free acid)
10.95 g	ZnSO ₄ .7H ₂ O
5.0 g	FeSO ₄ .7H ₂ O
1.54 g	MnSO ₄ .H ₂ O

392 mg	CuSO ₄ ·5H ₂ O
250 mg	Co(NO ₃) ₂ ·6H ₂ O
177 mg	Na ₂ B ₄ O ₇ ·10H ₂ O

Few drops of conc. H₂SO₄ was added to retard precipitation and adjusted to a final volume of one liter.

5.2.10 M9-minimal medium

5xM9 Salt	200 mL (prepared as described below)
1M MgSO ₄	2 mL (Autoclaved at 121°C for 20 minutes)
20% Glucose	20 mL (Autoclaved at 121°C for 20 minutes)
1M CaCl ₂	0.1 mL (Autoclaved at 121°C for 20 minutes)
750 mL distilled water autoclaved at 121°C for 20 minutes	

5xM9 Salt preparation

85.5 g	Na ₂ HPO ₄ ·12H ₂ O
15.0 g	KH ₂ PO ₄
2.5 g	NaCl
5.0 g	NH ₄ Cl
Deion. H ₂ O	1000 mL (Autoclaved at 121°C for 20 minutes)

5.2.11 Stock solutions

Reagent	Concentration in deionized H ₂ O
Amp	50 mg mL ⁻¹
Kan	50 mg mL ⁻¹
Chl	34 mg mL ⁻¹ in EtOH
IPTG	200 mg mL ⁻¹ (839 mM)
Glucose	20% w/v

Dissolve in deion. H₂O, sterilization by filtration (0.2µm).

5.3 Preparation of frozen stocks and cell cultures

5.3.1 *E. coli* JM109 (pDTG601A) expressing TDO

TDO genes originated from *P. putida* F1.^{208,81} LB agar plates were prepared with ampicillin (150 mg L⁻¹). Precultures were grown in LB medium or M9 Mineral Medium. Cultures were grown at 30°C in modified MSB medium containing glucose (10 mM), thiamine (0.1 mM), and ampicillin (150 µg mL⁻¹). The growing culture was induced with IPTG (100 µg mL⁻¹) after O.D. reached between 0.6 and 1.5 at 600 nm. At O.D. 2.0-2.5 cells were centrifuge off, washed twice with phosphate buffer and resuspended in MSB medium with addition of required components and the substrate 0.5-1 mg mL⁻¹ medium.

5.3.2 *E. coli* JM109 (DE3)(pDTG141) expressing NDO (NDO1)

The pDTG141 plasmid carries the *nahAaAbAcAd* genes encoding naphthalene dioxygenase from *pseudomonas species* NCIB 9816-4.²⁰⁹ The genes are under the control of T7 promoter pT7-5 in pDTG141 and can be induced by an additional IPTG. LB agar plates were prepared with ampicillin (150 mg per L). Precultures were grown in LB medium or M9 Mineral Medium. Cell cultures and induction was carried out under same condition as mentioned for TDO in section 5.3.1.

5.3.3 *E. coli* JM109 (PVL1343-PMS13) expressing NDO (NDO2)

E. coli JM109 recombinant strain carries the naphthalene dioxygenase (NDO) from *Pseudomonas fluorescence* N3. The plasmid pVL1343 contains the NDO genes (4.0 kb) and the wild type promoter cloned in the pVLT33 vector (kanamycin resistant, de Lorenzo *et al* 1993).²¹⁰ The plasmid PMS13 carrying the regulatory gene inducible by salicylate cloned in the pMS13 vector (ampicillin resistance). The strain is maintained on LB medium plates supplemented with ampicillin (100 µg mL⁻¹) and kanamycin (50 µg mL⁻¹). LB agar was prepared and autoclaved at 121°C for 20 minutes and plates were prepared after adding ampicillin (100 µg mL⁻¹) and kanamycin (50 µg mL⁻¹) at 37°C. Precultures with single colonies were grown in LB medium or mineral M9 medium at 30°C. Cultures were grown at 30°C in modified MSB medium containing glucose (0.2%), thiamine (1 µg mL⁻¹), ampicillin

²⁰⁸ [208] G. J. Zylstra and D. T. Gibson, *Genet. Eng.* **1991**, *13*, 183-203.

²⁰⁹ [209] S. M. Resnick, K. Lee and D. T. Gibson, *J. Ind. Microbiol. Biotechnol.* **1996**, *17*, 438-457.

²¹⁰ [210] V. de Lorenzo, L. Eltis, B. Kessler and K. N. Timmis, *Gene* **1993**, *123*, 17-24.

(100 $\mu\text{g mL}^{-1}$) and kanamycin (50 $\mu\text{g mL}^{-1}$). Induction was done with salicylic acid 2 mM (27.63 mg/100 mL medium) at O.D. between 0.6 and 1.5 at 600 nm. At O.D. 2.0-2.5 centrifuge off the cells, wash twice and resuspend in MSB medium with addition of required components and the substrate. Naphthalene was converted at about 2g L⁻¹ of medium. Sello *et al* showed that induction with salicylic acid increased the diol production 2-3 times more than with IPTG.²¹¹

5.4 Method optimization for detection of products

During screening scale biotransformations it was tested whether the substrate was accepted by the growing cells and how much was the relative ratio of product formation. It was also observed how many products were formed and if it was possible to resolve them on HPLC and GCMS. As highly polar and water soluble dihydroxylated products were formed, there was considerable difference in polarity between starting materials and products.

5.4.1 HPLC Method optimization for substrate conversion

HPLC analyses were recorded on a Thermo Finnigan Surveyor Plus using a reverse phase Phenomenex Luna C18 (2) column (10 μ , 250mmx4.6mm diameter). Detection was performed on a Thermo Finnigan PDA Plus photodiode array detector. (Standard method: 1mL/min, H₂O (0.1%TFA)/ACN = 85:15 1min → H₂O (0.1%TFA)/ACN = 20:80 9min → H₂O (0.1%TFA)/ACN = 20:80 3min → H₂O (0.1%TFA)/ACN = 85:15 3min → H₂O (0.1%TFA)/ACN = 85:15 5min). It was possible to correlate the rate of conversion to the peak area of starting material and product. It was also possible to find the relative percentage if more than one dihydroxylated products were formed.

5.4.2 GCMS Method optimization for substrate conversion

General conversion control was performed with GC Top 8000 / MS Voyager (quadropole, EI+) or GC Focus / MS DSQ II (quadropole, EI+) using a standard capillary column BGB5 (30mx0.32mm ID). Mass peaks of dihydroxylated products and starting materials were analyzed and conversions were measured as compared to starting materials.

²¹¹ [211] P. Di Gennaro, E. Galli, G. Albin, F. Pelizzoni, G. Sello and G. Bestetti, *Research in Microbiology* **1997**, 148, 355-364.

5.5 General procedures

This section gives general descriptions of repeatedly used protocols within this work in both chemical synthesis and biotransformation experiments.

5.5.1 Screening scale biotransformation with *Ralstonia eutropha* B9 (GP1)

HMB (20 mL) containing potassium succinate solution (112 μ L of 1.5M solution) was inoculated with 20 μ L of glycerol suspension. The culture was incubated at 120 rpm at 25°C on an orbital shaker for 2-3 days. An aliquot (1mL) of the cellular suspension was then transferred into 100-200 mL of HMB containing sodium succinate solution (5mM final concentration) and was again shaken at 120 rpm at 30°C on an orbital shaker for 2-3 days.

Without induction (GP1a):

Substrate (0.5-1mg) and potassium succinate solution (1.7 μ L per mL media; 1.5M) were added to the cellular suspension and the culture was incubated for 24-48 hours at 30°C.

With induction (GP1b):

Sodium benzoate (1M, 3mM final concentration) and potassium succinate solution (1.7 μ L per mL media; 1.5M) were added to the cellular suspension aqueous and shaken at 120 rpm at 25°C on an orbital shaker for 6 hours (TLC control). The cells were separated from the media by centrifugation (4000rpm, 4°C), the supernatant was discarded, and the cells were resuspended in the same amount of HMB. To the cellular suspension substrate (0.5-1mg per mL) and potassium succinate solution (1.7 μ L per mL media; 1.5M) were added and the culture was incubated for 24-48 hours.

Work-up:

Conversion was determined by TLC or/and HPLC. The biomass was removed by centrifugation (10000rpm, 4°C). The supernatant was concentrated under reduced pressure to 5-10% of its original volume at 40°C. The concentrate was cooled to 0°C and acidified to pH 3 using conc. HCl. The acidified aqueous solution was extracted repeatedly with EtOAc. The combined organic layers were dried over Na₂SO₄ and the solvent was evaporated at reduced pressure at 40°C.

5.5.2 Screening scale biotransformation (GP2)

Cells were streaked onto LBamp plate from frozen stocks of *E. coli* JM109 (DE3) containing TDO and incubated at 37°C for 12 h. A sterilized culture flask containing LBamp was inoculated with a single colony of *E. coli* JM109 (DE3).

The preculture was grown on an orbital shaker at 37 °C for 12 h at 180 rpm. In order to choose the right medium for biotransformation, experiments were carried out in M9 minimal medium and also in MSB Medium. Both M9 Mineral and MSB media (100 mL) containing 3 g L⁻¹ thiamine, 3 mL L⁻¹ ampicillin stock and 9.1 mL L⁻¹ of sterile 20% glucose solution, was transferred into 500 mL sterile baffled shake flask and was inoculated with 1 mL of preculture.

The cultures were grown at 30 °C until the optical density (OD) reached 1.5 to 2.0 at 660 nm. Cells were induced with 100 µL of IPTG stock solution and were grown for 2.5 h under same conditions. Culture was centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The supernatant was decanted and cells were resuspended in fresh M9 minimal medium and MSB medium containing 300 mg L⁻¹ thiamine, 3 mL L⁻¹ ampicillin stock and 9.1 mL 20% glucose solution.

An aliquot amount (0.5-1mg per mL) of substrate was added as solution in 1,4-dioxane. Samples (1 mL) were taken every 12 hrs in an Eppendorf's tube. Cells were removed by centrifugation and sample was analyzed by HPLC. In order to prepare samples for GCMS it was mixed with 0.5 mL of basic EtOAc and stirred rigorously. Organic layer was separated, dried over Na₂SO₄, and submitted for GCMS analysis. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (24-48 h).

As OD is in direct proportion with cellular activity, it was observed that generally higher OD (1.8 – 2.5) favored higher conversion rates. Biotransformations carried out in M9 mineral medium resulted in generally no to very low conversion of substrates whereas in MSB high conversions were observed. Therefore, MSB medium was selected as medium of choice for biotransformations. During screening scale biotransformations it was observed if the substrate was accepted by the growing cells and how much was relative ratio of product formation. It was also observed as how many products were formed and if it was possible to resolve them on HPLC and GCMS.

5.5.3 Preparative scale biotransformation with TDO (GP3)

Cells were streaked onto LBamp plate from frozen stocks of *E. coli* JM109 (DE3) containing TDO and incubated at 37°C for 12 h. Single colonies were selected for preculture preparation. A sterilized culture flask containing 10 mL of LBamp medium was inoculated with a single colony of *E. coli* JM109 (DE3) and the preculture was grown on an orbital shaker at 37 °C for 12 h at 180 rpm. MSB medium (200 mL) containing 3 g L⁻¹ thiamine, 3 mL L⁻¹ ampicillin stock and 9.1 mL L⁻¹ of 20% glucose solution, was transferred into 1 L sterile baffled shake flask and was inoculated with 2 mL of preculture and resulting culture was grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with 100 µL of IPTG and were grown for 2.5 h under same conditions. Culture was centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The supernatant was decanted and cells were resuspended in fresh MSB medium containing 300 mg L⁻¹ thiamine, 3 mL L⁻¹ ampicillin stock and 9.1 mL 20% glucose solution. An aliquot amount (0.5-1mg per mL) of substrate was added as solution in 1,4-dioxane. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-36 h).

Work-up:

After completion of biotransformation, the biomass was removed by centrifugation at 4500 rpm (3736 g) for 15 min at 4°C. The supernatant was concentrated at 30 °C to 5-10% of its original volume under reduced pressure. The concentrate was stirred with acid free EtOAc for 30-60 minutes. Acid free EtOAc was prepared by stirring it with saturated solution of Na₂CO₃ at low temperature. Organic layer was separated and the concentrate was again extracted with EtOAc multiple times. The combined organic layers were dried over Na₂SO₄ and the solvent was evaporated under reduced pressure at 40°C. The crude material was analyzed by ¹HNMR. Purification was performed by using either an automated MPLC with fraction collector or by manual flash column chromatography.

5.5.4 Preparative scale biotransformation with NDO1 (GP4)

Cells were streaked onto LBamp plate from frozen stocks of *E. coli* JM109 (DE3) containing NDO and incubated at 37°C for 12 h. Single colonies were selected for preculture preparation. A sterilized culture flask containing 10 mL of LBamp medium was inoculated with a single colony of *E. coli* JM109 (DE3) and the preculture was grown on an orbital

shaker at 37 °C for 12 h at 180 rpm. 200 mL of MSB medium containing 3 g L⁻¹ thiamine, 3 mL L⁻¹ ampicillin and 9.1 mL 20% glucose, was transferred into 1 L sterile shake flask and was inoculated with 2 mL of preculture and resulting culture was grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with 100 µL of IPTG and were grown for 2.5 h under same conditions. Culture was centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The supernatant was decanted and cells were resuspended in fresh MSB medium containing 3 g L⁻¹ thiamine, 3 mL L⁻¹ ampicillin and 9.1 mL 20% glucose. An aliquot amount of substrate was added as a solution in 1,4-dioxane. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-24 h).

Work-up was performed as described in GP3.

5.5.5 Preparative scale biotransformations with NDO2 (GP5)

Cells were streaked onto LBamp plate from frozen stocks of *E. coli* JM109 (PVL 1343 + PMS13) containing NDO2 and incubated at 37°C for 12 h. Single colonies were selected for preculture preparation. A sterilized culture flask containing 10 mL of LBamp+kan medium was inoculated with a single colony of *E. coli* JM109 (PVL 1343 + PMS13) and the preculture was grown on an orbital shaker at 37 °C for 12 h at 180 rpm. 200 mL of MSB medium containing 300 mg L⁻¹ thiamine, 4 mL L⁻¹ ampicillin, 2 mL L⁻¹ kanamycin and 20 mL 20% glucose, was transferred into 1 L sterile shake flask and was inoculated with 2 mL of preculture and resulting culture was grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with salicylic acid (as dioxane solution, 2mM L⁻¹ of MSB) and were grown for 2.5 h under same conditions. Culture was centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The supernatant was decanted and cells were resuspended in fresh MSB medium containing 300 mg L⁻¹ thiamine, 4 mL L⁻¹ ampicillin, 2 mL L⁻¹ kanamycin and 20 mL 20% glucose. An aliquot amount of substrate was added as a solution in 1,4-dioxane. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-24 h).

Work-up was performed as described in GP3.

5.5.6 Large scale shake flask biotransformation with NDO1 (GP6)

Cells were streaked onto LBamp plate from frozen stocks of *E. coli* JM109 (DE3) containing NDO1 and incubated at 37°C for 12 h. Single colonies were selected for preculture preparation. A sterilized culture flask containing 20 mL of LBamp medium was inoculated with a single colony of *E. coli* JM109 (DE3) and the preculture was grown on an orbital shaker at 37 °C for 12 h at 180 rpm. 2 L of MSB medium containing 300 mg L⁻¹ thiamine (600 mg), 3 mL L⁻¹ ampicillin (6 mL) and 9.1 mL L⁻¹ 20% glucose (18.2 mL) was inoculated with 20 mL of preculture. Resulting culture was distributed into 10x sterile shake flasks of 1 L capacity each containing 200 mL and was grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with 100 µL of IPTG in each flask and were grown for 2.5 h under same conditions. Cultures were centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The supernatant was decanted and combined cells were resuspended in 2L fresh MSB medium containing 300 mg L⁻¹ thiamine, 3 mL L⁻¹ ampicillin and 9.1 mL L⁻¹ 20% glucose. An aliquot amount of substrate was added as a solution in 1,4-dioxane and resulting culture was distributed in 10x sterile 1L shake flasks and grown on an orbital shaker at 37 °C. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-24 h).

After completion of reaction the cultures from all flasks were combined and work-up was performed as described in GP3.

5.5.7 Large scale shake flask biotransformation with NDO2 (GP7)

Cells were streaked onto LBamp plate from frozen stocks of *E. coli* JM109 (PVL 1343 + PMS13) containing NDO2 and incubated at 37°C for 12 h. Single colonies were selected for preculture preparation. A sterilized culture flask containing 20 mL of LBamp medium was inoculated with a single colony of *E. coli* JM109 (PVL 1343 + PMS13) and the preculture was grown on an orbital shaker at 37 °C for 12 h at 180 rpm. 2 L of MSB medium containing 30 mg L⁻¹ thiamine (60 mg), 4 mL L⁻¹ ampicillin (8 mL), 2 mL L⁻¹ kanamycin (4 mL), and 20 mL L⁻¹ 20% glucose (40 mL) was inoculated with 20 mL of preculture. Resulting culture was distributed into 10x sterile shake flasks of 1 L capacity each containing 200 mL and was grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with salicylic acid (as dioxane solution, 2mM L⁻¹ of MSB) in each flask and were grown for 2.5 h under same conditions. Cultures were centrifuged at 4500 rpm (3736 g) for 15 min at

4°C and supernatant was separated from cells. The supernatant was decanted and combined cells were resuspended in 2L fresh MSB medium containing 30 mg L⁻¹ thiamine, 4 mL L⁻¹ ampicillin, 2 mL L⁻¹ kanamycin, and 20 mL L⁻¹ 20% glucose. An aliquot amount of substrate was added as a solution in 1,4-dioxane and resulting culture was distributed in 10x sterile 1L shake flasks and grown on an orbital shaker at 37 °C. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-24 h).

After completion of reaction the cultures from all flasks were combined and work-up was performed as described in GP3.

5.5.8 Procedure for 2L scale fermentation with NDO1 (GP8)

Larger scale biotransformations were also carried out in New Brunswick Bioflow 110 Fermenter equipment having 2L capacity. The fermenter glassware was fitted with 0.2 μ syringe filters at the inlets and made airtight by closing all the valves were sealed with rubber tubing. The completely air tight setup of fermenter was autoclaved at 120°C for 20 minutes. The autoclaved fermenter was opened in Laminar Flow cabinet and 1.8 L initial volume of MSB medium was transferred to it and sealed again. The fermenter glassware apparatus was attached to the temperature control unit and inlets and outlets for water, oxygen, and temperature sensor were connected. The temperature was set at 30° C and agitation was set at 300 rpm. Continuous airflow was maintained at 3 L per minute. 20% glucose solution was used as carbon source at 10 mL L⁻¹. 300 mg L⁻¹ thiamine (600 mg), 3 mL L⁻¹ ampicillin (6 mL) and 10 mL L⁻¹ 20% glucose (20 mL) were added respectively through the inlet via a syringe filter. Once all the parameters were set, the medium was inoculated with 20 mL of preculture via syringe filter. The cells were grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with 2 mL of IPTG and were grown for 2.5 h under same conditions. After 2.5 h the agitation was stopped, fermenter was removed and culture was centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The combined cells were resuspended in 1.8 L fresh MSB medium and transferred to the fermenter. Thiamine 600 mg, ampicillin 6 mL, and 20% glucose 20 mL was added to the medium. An aliquot amount of substrate was added as a solution in 1,4-dioxane and reaction was performed at 30°C under conditions mentioned earlier. 1M HCl and 1M NaOH solutions were used to keep the pH range slightly basic at 7.3. Antifoam was added when necessary in order to avoid excessive foam production. Conversion of substrate was monitored with HPLC

and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-24 h).

Work-up:

After completion of biotransformation, the medium was transferred from fermenter to the centrifuge flasks. The biomass was removed by centrifugation at 4500 rpm (3736 g) for 15 min at 4°C. The supernatant was concentrated at 30 °C to 500 mL under reduced pressure. The concentrate was stirred with 500 mL acid free EtOAc for 60 minutes. Acid free EtOAc was prepared by stirring it with saturated solution of Na₂CO₃ at low temperature. Organic layer was separated and the concentrate was again extracted with 500 mL EtOAc three times. The combined organic layers were dried over Na₂SO₄ and the solvent was evaporated under reduced pressure at 40°C. The crude material was analyzed by ¹HNMR. Purification was performed by using either an automated MPLC with fraction collector or by manual flash column chromatography.

5.5.9 Procedure for 2L scale fermentation with NDO2 (GP9)

Larger scale biotransformations were also carried out in New Brunswick Bioflow 110 Fermenter equipment having 2L capacity. The fermenter glassware was fitted with 0.2 μ syringe filters at the inlets and made airtight by closing all the valves were sealed with rubber tubing. The completely air tight setup of fermenter was autoclaved at 120°C for 20 minutes. The autoclaved fermenter was opened in Laminar Flow cabinet and 1.8 L initial volume of MSB medium was transferred to it and sealed again. The fermenter glassware apparatus was attached to the temperature control unit and inlets and outlets for water, oxygen, and temperature sensor were connected. The temperature was set at 30° C and agitation was set at 300 rpm. Continuous airflow was maintained at 3 L per minute. 20% glucose solution was used as carbon source at 20 mL L⁻¹. 30 mg L⁻¹ thiamine (60 mg), 4 mL L⁻¹ ampicillin (8 mL), 2 mL L⁻¹ kanamycin (4mL) and 20 mL L⁻¹ 20% glucose (40 mL) were added respectively through the inlet via a syringe filter. Once all the parameters were set, the medium was inoculated with 20 mL of preculture via syringe filter. The cells were grown at 30 °C until the culture turbidity reached 1.5 to 2.0 at 660 nm. Cells were induced with salicylic acid (as dioxane solution, 2M L⁻¹ of MSB) and were grown for 2.5 h under same conditions. After 2.5 h the agitation was stopped, fermenter was removed and culture was centrifuged at 4500 rpm (3736 g) for 15 min at 4°C and supernatant was separated from cells. The combined cells were resuspended in 1.8 L fresh MSB medium and transferred to the fermenter. Thiamine 60

mg, ampicillin 8 mL, kanamycin 4 mL and 20% glucose 40 mL was added to the medium. An aliquot amount of substrate was added as a solution in 1,4-dioxane and reaction was performed at 30°C under conditions mentioned earlier. 1M HCl and 1M NaOH solutions were used to keep the pH range slightly basic at 7.3. Antifoam was added when necessary in order to avoid excessive foam production. Conversion of substrate was monitored with HPLC and the biotransformation was stopped when HPLC showed no further decrease in substrate peak or increase in product peak (18-24 h).

Work-up:

After completion of biotransformation, the medium was transferred from fermenter to the centrifuge flasks. The biomass was removed by centrifugation at 4500 rpm (3736 g) for 15 min at 4°C. The supernatant was concentrated at 30 °C to 500 mL under reduced pressure. The concentrate was stirred with 500 mL acid free EtOAc for 60 minutes. Acid free EtOAc was prepared by stirring it with saturated solution of Na₂CO₃ at low temperature. Organic layer was separated and the concentrate was again extracted with 500 mL EtOAc three times. The combined organic layers were dried over Na₂SO₄ and the solvent was evaporated under reduced pressure at 40°C. The crude material was analyzed by ¹HNMR. Purification was performed by using either an automated MPLC with fraction collector or by manual flash column chromatography.

5.5.10 Catalytic hydrogenation of diols (GP10)

Small scale catalytic hydrogenation was carried out using H₂ via needle and balloon at room temperature and an ambient pressure H₂ atmosphere. The palladium catalyst (10% Pd on C; 10% w/w) was added into the flask. Dry EtOAc (10 mL) was added under N₂ to a 2-necked 25 mL flask. Unsaturated diol was added and dissolved in dry EtOAc. Then, the reaction vessel was purged with H₂ (from a three layered balloon) and the reaction was stirred at room temperature under atmospheric pressure. Product formation was monitored by GCMS. After complete conversion, the suspension was filtered through a pad of Celite[®]. In most of the cases the yield of the reaction was quantitative without any requirement for further purification. If further purification was necessary, the solvent was evaporated under vacuum and product was purified by MPLC (silica gel, 100% EtOAc).

Large scale catalytic hydrogenation was carried out using a Parr apparatus. Unsaturated diol was added to 250 ml Paar flask and dissolved in about 80 mL dry EtOAc. The palladium catalyst (10% Pd on C; 10% w/w) was added into the flask. The flask was

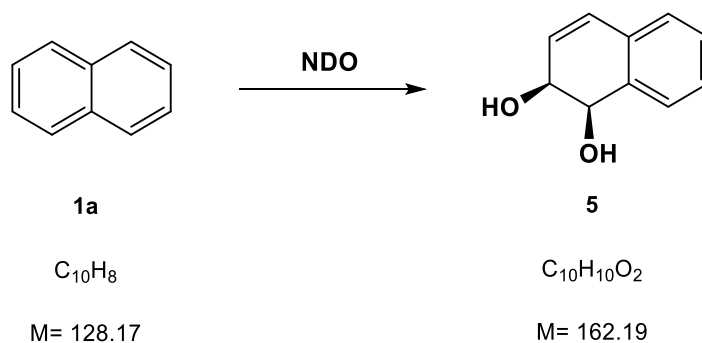
loaded to the Paar apparatus and purged with H₂ twice and then sealed under H₂ environment. The hydrogenation was carried out using a Parr apparatus under hydrogen pressure of approx. 60 PSI. Product formation was monitored by GCMS. After complete conversion, the suspension was filtered through a pad of Celite®. In most of the cases the yield of the reaction was quantitative without any requirement for further purification. If further purification was necessary, the solvent was evaporated under vacuum and product was purified by MPLC (silica gel, 100% EtOAc).

5.5.11 Cyclic carbonate formation of diols (GP11)

In order to synthesize the cyclic carbonate of reduced diol it was dissolved in dry DCM. The reaction was cooled to -10 °C in an ice bath under N₂ atmosphere. First, triethylamine (3.3-5.5 eq) was added to the reaction dropwise and then freshly distilled trichloroacetyl chloride (2.2 eq) diluted with DCM was slowly added dropwise. The reaction was stirred at -10 °C for 30 minutes and then slowly warmed to room temperature and stirred for 6 hours at that temperature. After completion of reaction, DCM was evaporated under reduced pressure, water was added and the reaction mixture was extracted three times with EtOAc. The organic layer was washed with brine and dried over Na₂SO₄ and the solvent was evaporated. Cyclic carbonate was purified by MPLC using EtOAc and hexane gradient mixture as eluent.

5.6 Preparative scale biotransformations

5.6.1 (+)-*cis*-(1R,2S)-1,2-Dihydronaphthalene-1,2-diol (**5**)



Biotransformation of naphthalene (**1a**) (3.00 g, 23.406 mmol) was carried out in 2L MSB medium distributed in 1L baffled shake flasks each of them containing 200 mL of MSB medium. Biotransformation was carried out using NDO2 as given in GP8. Conversion of starting material was monitored by HPLC in all shake flasks which showed similar behavior. The product **5** was purified by MPLC using EtOAc and PE as solvents (silica gel, gradient chromatography, 20% EtOAc 10 min, 20%-50% EtOAc 20 min, 50% EtOAc 30 min).

Yield: 75.32% (2.86 g, 17.63 mmol)

Appearance: colorless to beige solid

m.p.: 114-116 °C (Lit. 114-116°C)^{212,213}

TLC: 0.40 (hexane: EtOAc, 3: 1)

$\alpha_D^{20} =$ +224 (c 0.997, CHCl₃) Lit. +190 (c 0.10, MeOH),⁸⁷ +242 (c=1.00 CHCl₃), and +220 (c=0.08 MeOH)¹⁰⁴

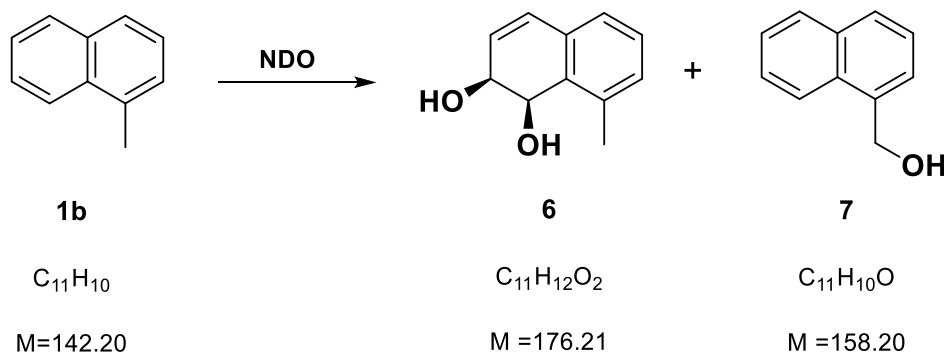
¹H NMR (200 MHz, CDCl₃): δ 4.34 – 4.42 (m, 1H, H2), 4.69 (d, J = 4.9 Hz, 1H, H1), 6.06 (dd, J = 9.7, 4.2 Hz, 1H, H3), 6.53 (d, J = 9.7 Hz, 1H, H4), 7.12 (dd, J = 5.4, 3.4 Hz, 1H, Ar-H), 7.28 – 7.32 (m, 2H, Ar-H), 7.53 (dd, J = 5.4, 3.5 Hz, 1H, AR-H).

²¹² [212] D. M. Jerina, J. W. Daly, A. M. Jeffrey and D. T. Gibson, *Archives of Biochemistry and Biophysics* **1971**, *142*, 394-396.

²¹³ [213] D. R. Boyd, N. D. Sharma, N. A. Kerley, R. A. S. McMordie, G. N. Sheldrake, P. Williams and H. Dalton, *Journal of the Chemical Society, Perkin Transactions 1* **1996**, 67-74.

^{13}C NMR (50 MHz, CDCl_3): δ 67.3 (d, C2), 70.0 (d, C1), 126.0 (d, C4), 126.6 (d, C8), 127.0 (d, C5), 127.3 (d, C6), 127.9 (d, C7), 128.4 (s, C4a), 132.1 (d, C3), 135.7 (s, C8a).

5.6.2 (+)-*cis*-(1R,2S)-8-Methyl-1,2-dihydronaphthalene-1,2-diol (**6**)



Biotransformation of 1-methylnaphthalene (**1b**) was carried out in MSB medium distributed in 1L baffled shake flasks containing 200 mL of MSB medium each. Substrate **1b** was dissolved in dioxane and evenly distributed in all shake flasks. Biotransformation was carried out using NDO1, NDO2, and TDO as given in respective general procedures. Total conversion was in range between 21 and 40 %. After completion of reaction the products were purified by MPLC using EtOAc and hexane as solvents (silica gel, gradient chromatography, EtOAc: PE, 0-20% EtOAc 20 min, 20%-50% EtOAc 20 min, 50% EtOAc 30 min). Spectral analysis of isolated products showed similar behavior of oxygenation as previously reported and both dioxygenated and monooxygenated products were isolated. Amount of substrate accepted and relative ratio of products obtained is given in Table 5.1.

Yield: 10-20%

Appearance: Colorless solid

m.p.: 114-116 °C (Lit. 112-113 °C)¹⁸¹

TLC: 0.40 (hexane: EtOAc, 3: 1)

$\alpha_{\text{D}}^{20} =$ +113 (c 1.01, CHCl_3) Lit. +97¹⁸¹ MeOH (c not available), +90.8 (c 1.0, MeOH).¹⁸⁰

^1H NMR (400 MHz, DMSO): δ 2.34 (s, 3H, CH_3), 4.26 – 4.34 (m, 1H, H2), 4.57 (d, J = 4.8 Hz, 1H, H1), 5.77 (d, J = 9.7 Hz, 1H, H3), 6.34 (dd, J = 9.7,

2.8 Hz, 1H, H4), 6.94 (d, J = 7.4 Hz, 1H, H6), 7.03 (d, J = 7.6 Hz, 1H, H7), 7.14 (t, J = 7.5 Hz, 1H, H5).

¹³C NMR (101 MHz, DMSO): δ 18.5 (q, CH₃), 65.4 (d, C1), 70.2 (d, C2), 124.8 (d, C4), 126.4 (d, C5), 128.3 (d, C6), 129.7 (d, C7), 132.8 (d, C3), 133.0 (s), 134.5 (s), 136.9 (s), (C4a, C1a, C8).

¹H NMR (200 MHz, CDCl₃): δ 2.41 (s, 3H, CH₃), 4.53 (dt, J = 4.8, 2.3 Hz, 1H, H2), 4.77 (dd, J = 5.1, 1.5 Hz, 1H, H1), 5.81 (dt, J = 9.8, 1.7 Hz, 1H, H3), 6.38 (dd, J = 9.8, 2.7 Hz, 1H, H4), 6.93 (d, J = 7.4 Hz, 1H, H6), 7.05 (d, J = 7.0 Hz, 1H, H7), 7.18 (t, J = 7.5 Hz, 1H, H5).

¹³C NMR (50 MHz, CDCl₃): δ 18.5 (q, CH₃), 66.5 (d, C1), 70.7 (d, C2), 125.3 (d, C4), 127.4 (d, C5), 129.3 (d, C6), 130.4 (d, C7), 130.9 (s), 132.0 (s), 137.5 (s), (C4a, C1a, C8).

5.6.3 Naphthalen-1-ylmethanol (7)

Yield: 4-13%

Appearance: beige solid

m.p.: 60-62 °C (Lit 58-63 °C)²¹⁴

TLC: 0.60 (hexane: EtOAc, 3: 1)

¹H NMR (200 MHz, CDCl₃): δ 5.09 (s, 2H), 7.37 – 7.61 (m, 4H), 7.76 – 7.95 (m, 2H), 8.02 – 8.16 (m, 1H).

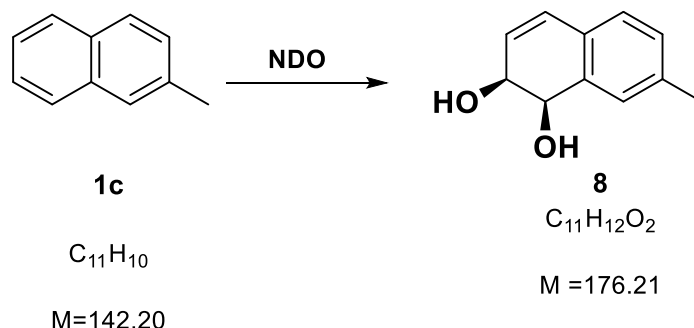
Table 5-1. 1-methylnaphthalene with different dioxygenases

	NDO1	NDO2	TDO
Substrate amount (g)	2.0	3.0	1.0
MSB Medium (L)	2	3	1
Conversion %	21	40	25
Yield 7 (mg, %)	5 % (100 mg)	13 % (427 mg)	8% (90 mg)

²¹⁴ [214] N. Misawa, M. Nodate, T. Otomatsu, K. Shimizu, C. Kaido, M. Kikuta, A. Ideno, H. Ikenaga, J. Ogawa, S. Shimizu and K. Shindo, *Applied Microbiology and Biotechnology* **2011**, *90*, 147-157.

Yield 6 (mg, %)	10% (230 mg)	20 % (732 mg)	14 % (168 mg)
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5.6.4 (+)-*cis*-(1*R*,2*S*)-7-Methyl-1,2-dihydronaphthalene-1,2-diol (**8**)



Biotransformation of 2-methylnaphthalene (**1c**) (200 mg, 1.41 mmol) was carried out in 600 mL MSB medium distributed in 3x1L baffled shake flasks each of them containing 200 mL of MSB medium. Biotransformation was carried out using NDO2 as given in GP5. Conversion of starting material was monitored by HPLC in all shake flasks which showed similar behavior. The product was purified by MPLC using EtOAc and hexane as solvents (silica gel, gradient chromatography, EtOAc: PE, 0-20% EtOAc 20 min, 20%-50% EtOAc 20 min, 50% EtOAc 20 min).

Yield: 48% (119 mg, 0.675 mmol)

Appearance: beige crystals

m.p.: 90-91 °C (Lit 89 °C)¹⁸¹

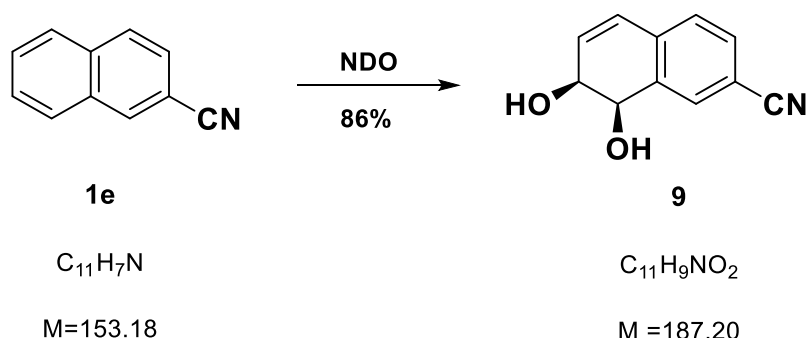
TLC: $R_f = 0.40$ (50%, EtOAc: hexane)

$\alpha_D^{20} = +197$ (c 0.93, $CHCl_3$) Lit. $+206^{181}$ MeOH(c not available)

1H NMR (200 MHz, $CDCl_3$): δ 2.36 (s, 3H, CH_3), 4.35 (t, $J = 4.3$ Hz, 1H, H2), 4.64 (d, $J = 4.9$ Hz, 1H, H1), 5.98 (dd, $J = 9.6$ Hz, 4.2 Hz, 1H, H3), 6.50 (d, $J = 9.7$ Hz, 1H, H4), 7.04 (dd, $J = 16.9, 7.7$ Hz, 2H, H5+H6), 7.34 (s, 1H, H8).

^{13}C NMR (50 MHz, $CDCl_3$): δ 21.6 (q, CH_3), 68.1 (d, C2), 70.9 (d, C1), 127.0 (d, C4), 127.6 (d, C8), 128.3 (d, C6), 129.2 (d, C5), 129.2 (d, C3), 134.8 (s, C4a), 138.2, (s, C7), 138.6 (s, C8a).

5.6.5 (+)-*cis*-(7*S*,8*R*)-7,8-Dihydroxy-7,8-dihydronaphthalene-2-carbonitrile (**9**)



Biotransformation of naphthalene-2-carbonitrile (**1e**) (2 g, 13.06 mmol) was carried out in 3L MSB medium distributed in 1L baffled shake flasks each of them containing 200 mL of MSB medium. Substrate **1e** was dissolved in dioxane and distributed in all flasks. Biotransformation was carried out using NDO2 as given in GP7. The product was purified by MPLC (silica gel, gradient chromatography, EtOAc: hexane, 30% EtOAc 20 min, 50% EtOAc 20 min, 50%-100% EtOAc 20 min, 100% EtOAc 20 min). It afforded one major dihydroxylated product with total yield of 86%. *cis*-(7*S*,8*R*)-7,8-dihydroxy-7,8-dihydronaphthalene-2-carbonitrile (**9**) was separated as colorless solid.

Yield: 86% (2.102 g, 11.23 mmol) (Lit. 76%)¹⁸³

Appearance: Colorless solid

m.p.: 121-123 °C (Lit. 102-105 °C)¹⁸³

TLC: $R_f = 0.40$ (EtOAc / hexane 1:1)

$[\alpha]_D^{20}$ +183 (c 0.97, $CHCl_3$), +272 (c 1.05 MeOH) Lit +215.6 (c 1.3, MeOH)¹⁸³

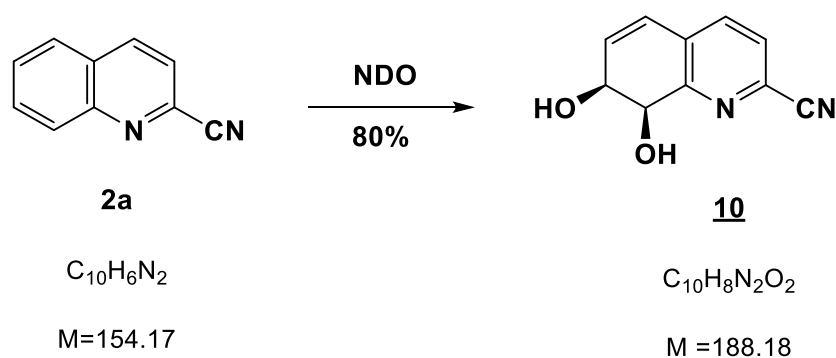
1H NMR (200 MHz, DMSO): δ 4.14 (dd, $J = 10.1, 5.1$ Hz, 1H, H7), 4.52 (t, $J = 5.5$ Hz, 1H, H8), 4.91 (d, $J = 5.7$ Hz, 1H, OH7), 5.34 (d, $J = 6.4$ Hz, 1H, OH8), 6.23 (dd, $J = 9.7, 4.8$ Hz, 1H, H6), 6.59 (d, $J = 9.7$ Hz, 1H, H5), 7.33 (d, $J = 7.8$ Hz, 1H, H4), 7.69 (dd, $J = 7.8, 1.4$ Hz, 1H, H3), 7.76 (s, 1H, H1).

¹³C NMR (101 MHz, DMSO): δ 65.5 (d, C7), 69.2 (d, C8), 109.4 (s, C2), 119.2 (s, CN), 126.6 (d, C4), 126.8 (d, C5), 129.5 (d), 131.3 (d), 134.0 (d), (C1, C3, C6), 137.0 (s), 139.2 (s), (C4a, C8a).

¹H NMR (200 MHz, CDCl₃): δ 4.37 (t, J = 4.9 Hz, 1H, H7), 4.72 (d, J = 4.9 Hz, 1H, H8), 6.27 (dd, J = 9.7, 4.8 Hz, 1H, H6), 6.59 (d, J = 9.7 Hz, 1H, H5), 7.20 (d, J = 7.8 Hz, 1H, H4), 7.55 (dd, J = 7.8, 1.6 Hz, 1H, H3), 7.83 (s, 1H, H1).

¹³C NMR (50 MHz, CDCl₃): δ 66.5 (d, C7), 69.9 (d, C8), 111.7 (s, C2), 118.9 (s, CN), 127.4 (d, C4), 128.6 (d, C5), 130.6 (d), 131.5 (d), 132.4 (d), (C1, C3, C6), 136.0 (s), 137.3 (s), (C4a, C8a).

5.6.6 (+)-*cis*-(7S,8R)-7,8-Dihydroxy-7,8-dihydroquinoline-2-carbonitrile (**10**)



Biotransformation of quinolone-2-carbonitrile (**2a**) (70 mg, 0.454 mmol) was carried out in 1L baffled shake flask containing 200 mL of MSB medium according to GP5. After completion of reaction cells were centrifuged off and supernatant was concentrated under reduced pressure, extracted several times with EtOAc. The collected EtOAc extracts were combined and dried over Na₂SO₄, filtered and solvent evaporated. The product was purified by MPLC (silica gel, 100% EtOAc). The *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroquinoline-2-carbonitrile (**10**) was recrystallized from EtOAc/hexane to afford a colorless to beige solid.

Yield: 80% (68 mg, 0.361 mmol)

Appearance: Colorless to beige solid

Elemental Analysis: Calc.: C 63.82%, H 4.28%, N 14.89%

Found: C 63.65%, H 4.24%, N 14.61%

m.p.: 148-151°C

TLC: $R_f = 0.60$ (EtOAc)

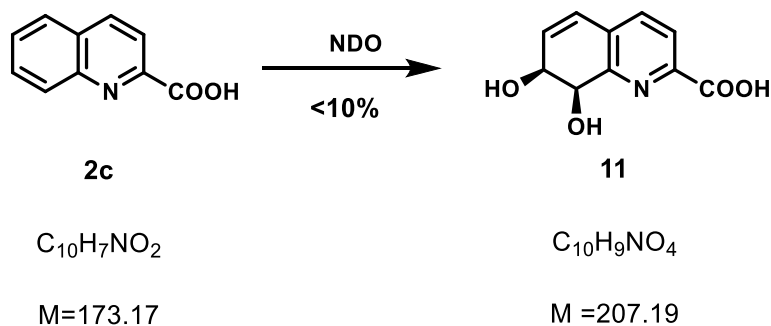
Specific rotation: $\alpha_D^{25} = +174$ ($c = 0.875$, EtOAc), $+224$ ($c = 1.0$, EtOAc)

$^1\text{H-NMR}$ (200MHz, CDCl_3): δ 4.52 (dd, $J = 5.8$ Hz, 4.9 Hz, 1H, H7), 4.78 (d, $J = 4.8$ Hz, 1H, H8), 6.48 (dd, $J = 9.7$ Hz, 5.8 Hz, 1H, H6), 6.72 (d, $J = 9.7$ Hz, 1H, H5), 7.55 (d, $J = 7.8$ Hz, 1H, H4), 7.64 (d, $J = 7.7$ Hz, 1H, H3).

$^1\text{H NMR}$ (400 MHz, DMSO-d_6): δ 4.41 (s, 1H, H7), 4.50 (d, $J = 4.7$ Hz, 1H, H8), 6.24 (dd, $J = 9.8$ Hz, 3.5 Hz, 1H, H6), 6.59 (dd, $J = 1.5$ Hz, 9.8 Hz, 1H, H5), 7.77 (d, $J = 7.8$ Hz, 1H, H4), 7.91 (d, $J = 7.8$ Hz, 1H, H3).

$^{13}\text{C NMR}$ (50MHz, DMSO-d_6): δ 68.0 (d, C7), 71.2 (d, C8), 118.3 (s, CN), 124.5 (d, C5), 129.2 (d, C3), 129.6 (s, C2), 132.2 (s, C4a), 134.4 (d, C4), 137.3 (d, C6), 159.2 (s, 8a).

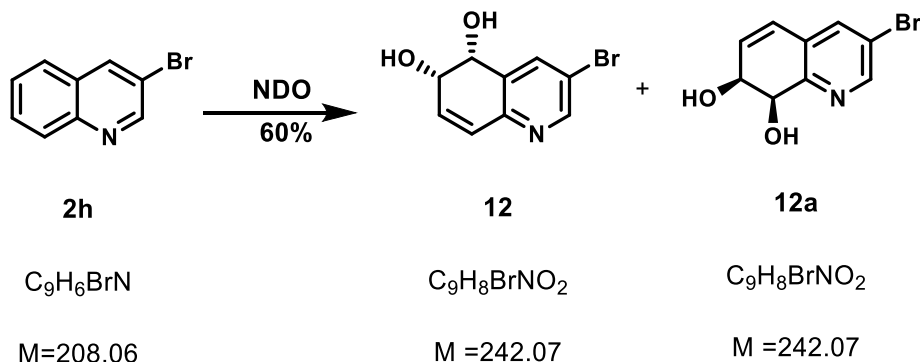
5.6.7 *cis*-7,8-Dihydroxy-7,8-dihydroquinoline-2-carboxylic acid* (11)



Biotransformation of quinolone-2-carboxylic acid (**2c**) (50 mg, 0.289 mmol) was carried out in 200 mL MSB medium in 1L baffled shake flask. Biotransformation was carried out using NDO2 as given in GP5. Conversion of starting material was monitored by HPLC which showed the formation of product and consumption of starting material. Crude NMR analysis showed formation of dihydroxylated product. Although dihydroxy diol was produced, it was extremely difficult to isolate it from the parent acid. Various unsuccessful attempts were made in order to recover the diol in pure form for analytical purpose. The assignment is tentative and is based on correlation with diols obtained from biotransformation of 2-substituted quinoline.

¹H NMR (200 MHz, DMSO): δ 4.37-4.45 (m, 1H), 4.48 (d, $J = 4.8$ Hz, 1H), 6.23 (dd, $J = 9.7, 3.5$ Hz, 1H), 6.59 (dd, $J = 9.8, 1.4$ Hz, 1H), *7.54 (m, 1H), *7.76 (m, 1H), *7.90 (m, 2H).

5.6.8 (+)-*cis*-(5R,6S)-3-Bromo-5,6-dihydroquinoline-5,6-diol (**12**)



Biotransformation of 3-bromoquinoline (**2h**) (626 mg, 3 mmol) was carried out in 1L MSB medium distributed in 1L baffled shake flasks each of them containing 200 mL of MSB medium. Biotransformation was carried out using NDO2 as described in GP7. Conversion of starting material was monitored by HPLC in all shake flasks which showed similar behavior. The product was purified by MPLC using EtOAc and hexane as solvents (silica gel, gradient chromatography, EtOAc: hexane, 0-20% EtOAc 20 min, 20-50% EtOAc 30 min, 50%-100% EtOAc 30 min, 100% EtOAc 20 min). Major fraction obtained was 5,6-diol **12** and minor unstable fraction was assigned as 7,8-diol **12a**.

Yield: 60% (436 mg, 1.80 mmol) Lit (23%)¹⁸⁴

Appearance: dark yellow crystalline solid

m.p.: 177-180 °C (Lit 171°C)

TLC: 0.4 (EtOAc: hexane, 4: 6)

Specific rotation $\alpha_D^{20} = +193$ ($c = 0.83$, EtOAc (Lit ¹⁸⁶+220 ($c = 0.69$, MeOH)

¹H NMR (200 MHz, DMSO): δ 4.12 (d, $J = 4.6$ Hz, 1H, H6), 4.57 (t, $J = 4.1$ Hz, 1H, H5), 4.97 (d, $J = 4.7$ Hz, 1H, OH), 5.40 – 5.53 (m, 1H, OH), 6.39 (dd, $J = 9.9$ Hz, 4.8 Hz, 1H, H7), 6.53 (d, $J = 9.9$ Hz, 1H, H8), 7.87 – 7.95 (m, 1H, H2), 8.48 (dd, $J = 2.4$ Hz, 0.8 Hz, 1H, H4).

¹H NMR (200 MHz, CDCl₃): δ 4.34 (t, $J = 5.2$ Hz, 1H, H6), 4.77 (d, $J = 4.4$ Hz, 1H, H5), 6.46 (dd, $J = 9.9$ Hz, 5.1 Hz, 1H, H7), 6.72 (d, $J = 9.9$ Hz, 1H, H8), 8.03 (d, $J = 1.7$ Hz, 1H, H2), 8.46 (d, $J = 2.2$ Hz, 1H, H4).

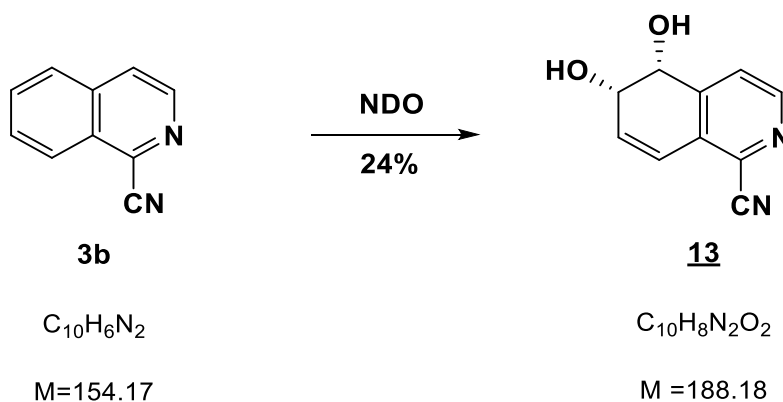
¹³C NMR (50 MHz, CDCl₃): δ 65.6 (d, C6), 69.6 (d, C5), 120.0 (s, C3), 131.0 (d, C8), 132.2 (d, C7), 133.9 (s, C4a), 137.2 (d, C4), 149.1 (d, C2), 149.5 (s, C8a).

5.6.9 (+)-*cis*-(7S,8R)-3-Bromo-7,8-dihydroquinoline-7,8-diol (**12a**)

Yield: *20% (145 mg, 0.60 mmol)

¹H NMR (200 MHz, CDCl₃): δ 4.46 (dd, $J = 6.2, 4.0$ Hz, 1H), 4.70 (d, $J = 4.9$ Hz, 1H), 6.33 (dd, $J = 9.5, 5.6$ Hz, 1H), 6.58 (d, $J = 9.7$ Hz, 1H), 8.30 (d, $J = 3.9$ Hz, 1H), 8.50 (d, $J = 2.1$ Hz, 1H).

5.6.10 *cis*-(5R,6S)-5,6-Dihydroxy-5,6-dihydroisoquinoline-1-carbonitrile (**13**)



Biotransformation of isoquinoline-1-carbonitrile (**3b**) (200 mg, 1.297 mmol) was carried out in four 1L baffled shake flasks each of them containing 200 mL of MSB medium. Substrate **3b** was distributed in four portions of 50 mg in all four flasks. Biotransformation

was carried out using NDO1 as given in GP4. Conversion of starting material was monitored by HPLC in all four flasks which showed similar behavior. Substrate conversion for isoquinoline-1-carbonitrile (**3b**) was relatively less favored hence lower concentration was used. After completion of reaction cells were centrifuged off and transformation media from all four flasks were combined. Supernatant was concentrated under reduced pressure, extracted several times with EtOAc. The collected EtOAc extracts were combined and dried over Na₂SO₄, filtered and solvent evaporated.

The product was purified by MPLC (45 g silica gel, gradient chromatography, EtOAc: hexane, 50% EtOAc 8 min, 50%-100% EtOAc 12 min, 100% EtOAc 20 min). It afforded two dihydroxylated products with total yield of 42%. *cis*-(5R,6S)-5,6-dihydroxy-5,6-dihydroisoquinoline-1-carbonitrile (**13**) was separated as colorless and relatively more polar fraction.

Yield: 24% (58 mg, 0.308 mmol)

Appearance: Colorless solid

α_D^{20} : +81 (c= 0.485, MeOH)

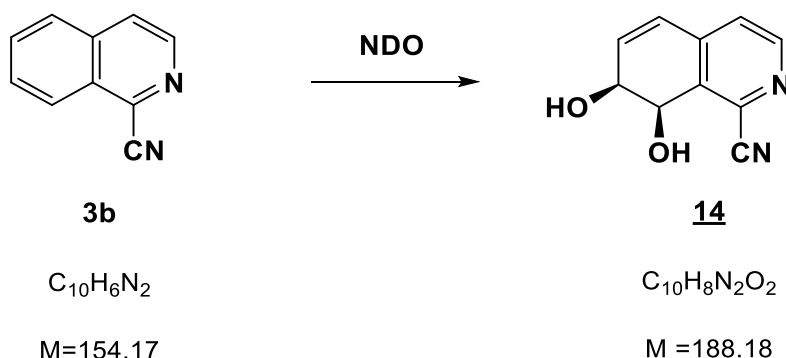
TLC: 0.40 (EtOAc)

¹H NMR (400 MHz, MeOD): δ 4.29 (t, J = 5.2 Hz, 1H, H6), 4.73 (d, J = 4.9 Hz, 1H, H5), 6.55 (dd, J = 9.8 Hz, 5.5 Hz, 1H, H7), 6.89 (d, J = 9.8 Hz, 1H, H8), 7.82 (d, J = 4.8 Hz, 1H, H4), 8.55 (d, J = 4.8 Hz, 1H, H3).

¹³C NMR (101 MHz, MeOD): δ 66.2 (d, C6), 70.6 (d, C5), 116.7 (s, CN), 123.7 (d, C8), 125.7 (d, C4), 129.9 (s, C1), 133.9 (s, C8a), 136.1 (d, C7), 149.9 (s, C4a), 151.2 (d, C3).

¹H NMR (200 MHz, CDCl₃): δ 4.19 – 4.26 (m, 1H, H6), 4.61 (d, J = 4.6 Hz, 1H, H5), 5.96 (d, J = 9.8 Hz, 1H, H7), 6.13 (dd, J = 9.8 Hz, 2.0 Hz, 1H, H8), 6.92 (d, J = 4.9 Hz, 1H, H4), 8.23 (d, J = 4.9 Hz, 1H, H3).

5.6.11 *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-1-carbonitrile (**14**)



Biotransformation of isoquinoline-1-carbonitrile (**3b**) (200 mg, 1.297 mmol) was carried out in four 1L baffled shake flasks each of them containing 200 mL of MSB medium. It afforded two dihydroxylated products with total yield of 42%. *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-1-carbonitrile (**14**) was separated as colorless and relatively less polar fraction.

Yield: 18% (45 mg, 0.239 mmol)

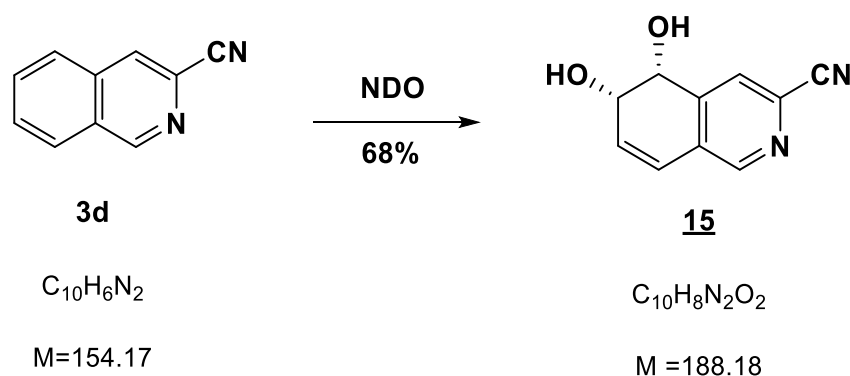
Appearance: Colorless solid

TLC: 0.50 (EtOAc)

1H NMR (400 MHz, MeOD): δ 4.57 (dt, $J=4.8$ Hz, 2.4 Hz, 1H, H7), 4.91 (dd, $J=4.9$ Hz, 1.0 Hz, 1H, H8), 6.24 – 6.33 (m, 1H, H6), 6.55 (dd, $J=9.8$ Hz, 2.6 Hz, 1H, H5), 7.40 (d, $J=4.9$ Hz, 1H, H4), 8.56 (d, $J=4.9$ Hz, 1H, H3).

^{13}C NMR (101 MHz, MeOD): δ 67.4 (d, C8), 70.3 (d, C7), 116.8 (s, CN), 124.7 (d, C5), 125.1 (d, C4), 134.5 (s, C1), 136.6 (s, C8a), 140.8 (d, C6), 142.8 (s, C4a), 152.6 (d, C3).

5.6.12 (+)-*cis*-(5R,6S)-5,6-Dihydroxy-5,6-dihydroisoquinoline-3-carbonitrile (**15**)



Biotransformation of isoquinoline-3-carbonitrile (**3d**) (55 mg, 0.357 mmol) was carried out in 1L baffled shake flask containing 200 mL of MSB medium according to GP2. The product was purified by MPLC (45 g silica gel, gradient chromatography, EtOAc: PE, 10% EtOAc 5 min, 10%-100% EtOAc 12 min, 100% EtOAc 15 min). NMR analysis showed formation of two products which were not separable by column chromatography. Yield was calculated from mixture of products (77%, total 52 mg, 0.276 mmol). Relative ratio of products according to NMR analysis was around 90:10 whereas *cis*-diol **15** was major product (46mg, 0.244 mmol). Major diol **15** was relatively more soluble in cold EtOAc and multiple extractions with cold EtOAc afforded >98% pure *cis*-diol **15**.

Yield: 68% (46 mg, 0.244 mmol) (calculated from mixture)

Appearance: colorless to beige solid

Elemental Analysis: Calc.: C 63.82%, H 4.28%, N 14.89%

Found: C 63.88%, H 4.13%, N 14.76%

m.p.: 204-206 °C (EtOAc: hexane)

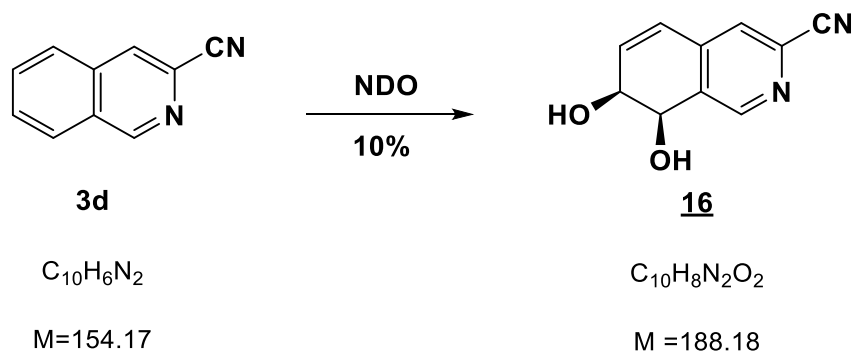
TLC: 0.50 (EtOAc: hexane, 8:2)

α_D^{20} = +240 (C= 1.02, EtOAc)

1H NMR (400 MHz, DMSO- d_6): δ 4.15 (t, J = 5.2 Hz, 1H, H6), 4.59 (d, J = 5.0 Hz, 1H, H5), 5.01 (bs, OH, OH6), 5.66 (bs, OH, OH5), 6.41 (dd, J = 9.7 Hz, 5.4 Hz, 1H, H7), 6.71 (d, J = 9.7 Hz, 1H, H8), 7.93 (s, 1H, H4), 8.54 (s, 1H, H1).

^{13}C NMR (50MHz, DMSO- d_6): δ 64.8 (d, C6), 69.3 (d, C5), 118.3 (s, CN), 124.0 (d, C8), 125.9 (d, C4), 131.5 (s, C3), 132.2 (s, C8a), 135.6 (d, C7), 148.0 (d, C1), 148.9 (s, C4a).

5.6.13 *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-3-carbonitrile (**16**)



cis-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-3-carbonitrile (**16**) was produced as minor product during the biotransformation of isoquinoline-3-carbonitrile (**3d**) (55mg, 0.357 mmol). Minor diol **16** was not isolated as 100% pure fraction from mixture of diols. After multiple cold extractions with EtOAc diol **15** was separated with >98% purity. After extractions diol **16** should be available as almost pure fraction, but it was found to be gummy substance which on NMR analysis showed strange signals pattern suggesting decomposition and was hence not separated as pure fraction.

Yield: 10% (6 mg, 0.032 mmol) (Calculated from mixture)

Appearance: colorless to beige solid

m.p.: was not isolated

TLC: 0.50 (EtOAc: hexane, 8:2)

$\alpha_{\text{D}}^{20} =$ was not isolated

¹H NMR (400 MHz, DMSO-d₆)*: δ 4.20 (t, J = 5.3 Hz, 1H, H7), 4.65 (dd, J = 10.3 Hz, 4.6 Hz, 1H, H8), 6.41 (dd, J = 9.7, 5.4, 1H, H6), 6.58 (d, J = 9.62, 1H, H5), 7.82 (s, 1H, H4), 8.72 (s, 1H, H1).

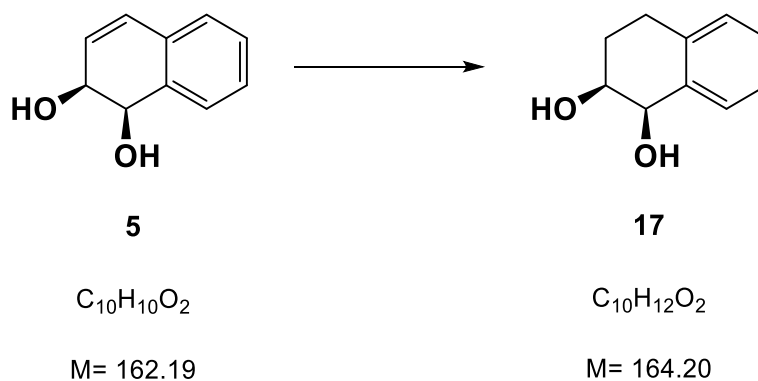
¹³C NMR (100MHz, DMSO-d₆)*: δ 65.2 (d, C7), 68.2 (d, C8), 117.4 (s, CN), 124.1, 124.9, 135.77, 137.2, 137.7, 140.9, 148.6 (d, C1).

¹H NMR (200 MHz, CDCl₃)*: δ 4.47 (t, J = 5.3 Hz, 1H, H7), 4.86 (dd, J = 10.3 Hz, 4.6 Hz, 1H, H8), 6.47 (dd, J = 9.7, 5.4, 1H, H6), 6.54 (d, J = 9.62, 1H, H5), 7.40 (s, 1H, H4), 8.82 (s, 1H, H1).

5.7 Reduction of diols

Reduction of diols obtained by biotransformation was carried out to convert them to saturated and hence more stable tetrahydro diols. This would eliminate the chances of water elimination and hence rearomatization of the diols. In most cases (for small quantities up to 200 mg) hydrogenation was carried out at room temperature and with the help of balloons. For larger quantities reduction was carried out in a Parr apparatus under high pressure 50-60 psi overnight.

5.7.1 (-)-*cis*-(1*R*,2*S*)-1,2,3,4-Tetrahydronaphthalene-1,2-diol (**17**)



(1*R*,2*S*)-1,2-dihydronaphthalene-1,2-diol (**5**) (1.25g, 7.71 mmol) was filled in the 250 mL Parr hydrogen flask. Dry EtOAc (70 mL) was added and 125 mg Pd catalyst (10 % w/w on charcoal) was added. Catalytic hydrogenation was carried according to GP10 at 50 PSI overnight. The catalyst was removed by filtration over Celite[®]. Then, the reaction solution was passed over a small column of silica and the solvent was evaporated.

Yield: 93% (1.18g, 7.19 mmol)^{215,216}

Appearance: colorless to beige solid

m.p.: 133-134°C (Lit 128-129°C,²¹⁷ 140-142°C²¹⁸ and 103-106 °C²¹⁹)

TLC: 0.3 (EtOAc: hexane, 40: 60)

²¹⁵ [215] V. Suresh, J. J. P. Selvam, K. Rajesh, V. Shekhar, D. C. Babu and Y. Venkateswarlu, *Synthesis* **2010**, 1763-1765.

²¹⁶ [216] M. Shibuya, T. Shibuta, H. Fukuda and Y. Iwabuchi, *Organic Letters* **2012**, *14*, 5010-5013.

²¹⁷ [217] K. Naemura, T. Wakebe, K. Hirose and Y. Tobe, *Tetrahedron: Asymmetry* **1997**, *8*, 2585-2595.

²¹⁸ [218] F. Orsini, G. Sello, E. Travaini and P. Di Gennaro, *Tetrahedron: Asymmetry* **2002**, *13*, 253-259.

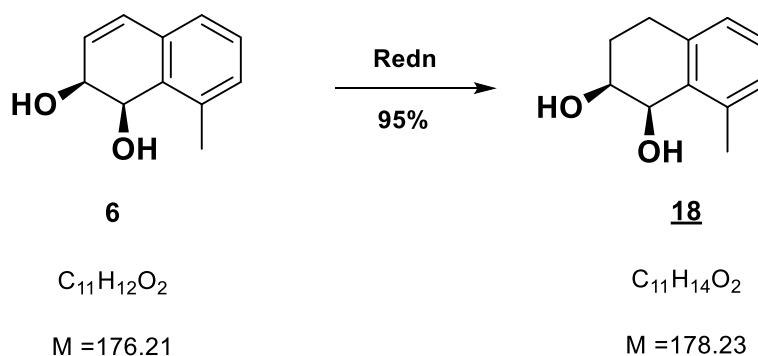
²¹⁹ [219] S. K. Balani, D. R. Boyd, E. S. Cassidy, G. I. Devine, J. F. Malone, K. M. McCombe, N. D. Sharma and W. B. Jennings, *J. Chem. Soc., Perkin Trans. 1* **1983**, 2751-2756.

α_D^{20} -64 (c=1.0, CHCl₃) Lit -38 (0.87, CHCl₃) and -41²¹⁹

¹H NMR (200 MHz, CDCl₃): δ 1.85 – 2.17 (m, 2H, H3), 2.70 – 2.89 (m, 1H, H4), 2.99 (dt, J = 17.1, 5.5 Hz, 1H, H4), 4.04 (dt, J = 9.6, 3.7 Hz, 1H, H2), 4.72 (d, J = 3.8 Hz, 1H, H1), 7.10 – 7.18 (m, 1H, Ar-H), 7.21 – 7.25 (m, 2H, Ar-H), 7.40 – 7.49 (m, 1H, Ar-H).

¹³C NMR (50 MHz, CDCl₃): δ 26.3 (t, C3), 27.1 (t, C4), 69.6 (d, C1), 70.1 (d, C2), 126.9 (d, Ar-C), 128.2 (d, Ar-C), 128.7 (d, Ar-C), 130.0 (d, Ar-C), 136.3 (s, C4a), 136.4 (s, C8a).

5.7.2 *cis*-(1R,2S)-8-Methyl-1,2,3,4-tetrahydronaphthalene-1,2-diol (**18**)



cis-(1R,2S)-8-methyl-1,2-dihydronaphthalene-1,2-diol (**6**) (400 mg, 2.27 mmol) was filled in the 250 mL Parr hydrogen flask. Dry EtOAc (70 mL) was added and 40 mg Pd catalyst (10 % w/w on charcoal) was added. Catalytic hydrogenation was carried according to GP7 at 50 PSI overnight. The catalyst was removed by filtration over Celite[®]. Then, the reaction solution was passed over a small column of silica and the solvent was evaporated. Compound **18** was purified by passing over small column of silica and evaporating the solvent.

Yield: 96% (388 mg, 2.18 mmol)

Appearance: colorless solid

m.p.: 168-169 °C

α_D^{20} : -78.5 (c 0.95, MeOH)

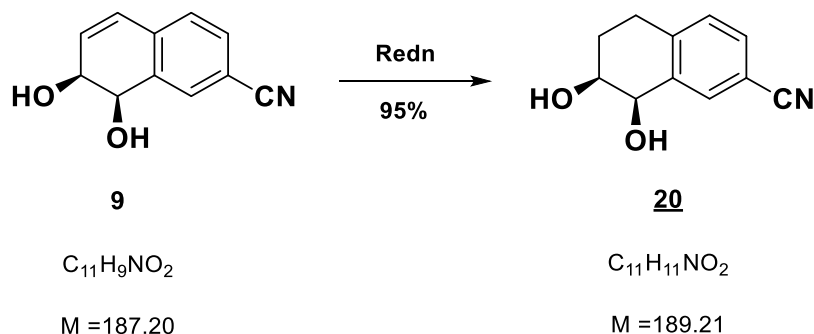
TLC: 0.40 (40% EtOAc: hexane)

¹H NMR (200 MHz, CDCl₃): δ 1.86 – 2.02 (m, 2H, H3), 2.47 (s, 3H, CH₃), 2.81 – 2.94 (m, 2H, H4), 3.80 – 3.93 (m, 1H, H2), 4.85 (t, J = 4.0 Hz, 1H, H1), 6.98 (d, J = 7.6 Hz, 1H, H7), 7.07 (d, J = 7.5 Hz, 1H, H5), 7.15 (t, 7.5 Hz, 1H, H6).

¹H NMR (400 MHz, DMSO): δ 1.57 – 1.70 (m, 1H, H3), 1.89 (qd, J = 12.1 Hz, 6.2 Hz, 1H, H3), 2.36 (s, 3H, CH₃), 2.64 – 2.85 (m, 2H, H4), 3.59 (ddt, J = 12.6 Hz, 6.5 Hz, 3.5 Hz, 1H, H2), 4.54 (d, J = 5.0 Hz, 1H, OH1), 4.58 (d, J = 3.9 Hz, 1H, H1), 4.64 (d, J = 5.9 Hz, 1H, OH2), 6.90 (d, J = 7.6 Hz, 1H, H7), 6.98 (d, J = 7.4 Hz, 1H, H5), 7.06 (t, J = 7.4 Hz, 1H, H6).

¹³C NMR (101 MHz, DMSO): δ 18.4 (q, CH₃), 24.9 (t, C3), 28.7 (t, C4), 66.0 (d, C1), 69.8 (d, C2), 126.0 (d, C7), 127.1 (d, C5), 127.4 (d, C6), 136.0 (s), 136.2 (s), 138.1 (s) (C4a, C8a, C8).

5.7.3 (+)-*cis*-(7S,8R)-7,8-Dihydroxy-5,6,7,8-tetrahydronaphthalene-2-carbonitrile (**20**)



cis-(7S,8R)-7,8-dihydroxy-7,8-dihydronaphthalene-2-carbonitrile (**9**) (1.5g, 8.01 mmol) was filled in the 250 mL Parr hydrogen flask. Dry EtOAc (70 mL) was added and 150 mg Pd catalyst (10 % w/w on charcoal) was added. Catalytic hydrogenation was carried according to GP7 at 50 PSI overnight. The catalyst was removed by filtration over Celite[®]. Then, the reaction solution was passed over a small column of silica and the solvent was evaporated. Tetrahydrodiol **20** was purified by passing over small column of silica and evaporating the solvent.

Yield: 98 % (1.485 g, 7.85 mmol)

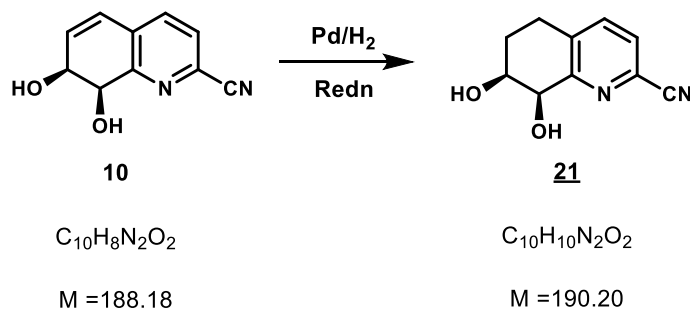
Appearance: colorless to greenish solid

Appearance: colorless solid
m.p.: 105-107 °C
 α_D^{20} : +29.1 (c 1.0, MeOH)
TLC: 0.40 (40% EtOAc: hexane)

^1H NMR (400 MHz, CDCl_3): δ 1.90 – 2.01 (m, 1H, H3), 2.13 (dq, $J = 13.9$ Hz, 7.1 Hz, 1H, H3), 2.80 (dt, $J = 17.9$ Hz, 6.7 Hz, 1H, H4), 3.00 – 3.15 (m, 1H, H4), 4.14 (dt, $J = 8.3$ Hz, 3.3 Hz, 1H, H2), 4.70 (d, $J = 3.8$ Hz, 1H, H1), 7.22 (d, $J = 7.9$ Hz, 1H, H5/H6), 7.48 (dd, $J = 7.9$ Hz, 1.8 Hz, 1H, H6/H5), 7.81 (s, 1H, H8).

^{13}C NMR (101 MHz, CDCl_3): δ 26.2 (t, C6), 26.4 (t, C5), 69.4, (d, C7), 77.2 (d, C8), 110.4 (s, C2), 119.1 (s, CN), 129.4 (d, C4), 133.4 (d, C1/C3), 138.0 (d, C1/C3), 139.4 (s, C4a), 142.1 (s, C8a).

5.7.4 (+)-cis-(7S,8R)-7,8-Dihydroxy-5,6,7,8-tetrahydroquinoline-2-carbonitrile (21)



Diol **10** (100 mg, 0.531 mmol) was filled in a 2-necked 25 mL flask. Dry EtOAc (10 mL) was added and 10 mg Pd catalyst (10 % w/w on charcoal) was added. Hydrogen gas filled in a three layered balloon was purged via syringe and needle and reaction was stirred at room temperature and ambient pressure. Catalytic hydrogenation was carried according to GP7 at 50 PSI overnight. Product formation was monitored by GCMS as both normal and reduced diol have almost same R_f value on the TLC. The catalyst was removed by filtration over Celite[®]. Then, the reaction solution was passed over a small column of silica and the solvent was evaporated. Tetrahydrodiol **21** was purified by MPLC (silica gel, 100% EtOAc).

Yield: 95% (96 mg, 0.504 mmol)

Appearance: Colorless crystals

Elemental Analysis: Calc.: C 63.15%, H 5.30%, N 14.73%
Found: C 63.90%, H 5.28%, N 14.08%

m.p.: 112-114°C (EtOAc: hexane)

TLC: 0.50 (EtOAc)

α_D^{20} : +87 (c= 0.565, CHCl₃)

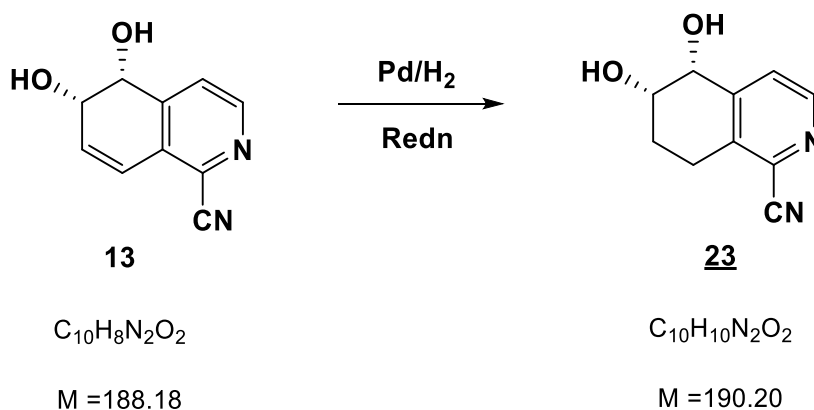
¹H NMR (200 MHz, DMSO): δ 1.95 (m, 2H, CH₂-6), 2.94 (dt, J = 14.5 Hz, 12.7 Hz, 2H, CH₂-5), 3.93 (dt, J = 9.1 Hz, 3.1 Hz, 1H, H7), 4.54 (s, 1H, H8), 4.83 (s, OH), 5.45 (s, OH), 7.81 (d, J = 8.0 Hz, 1H, H4), 7.89 (d, J = 7.9, 1H, H3).

¹³C NMR (50MHz, DMSO): δ 30.1 (t, C6), 30.6 (t, C5), 73.1 (d, C7), 75.7 (d, C8), 122.9 (s, CN), 132.5 (d, C3), 134.9 (s, C2), 142.2 (s, C4a), 143.0 (d, C4), 164.7 (s, C8a).

¹H NMR (400 MHz, CDCl₃): δ 1.96 (m, 1H, H6), 2.26 (m, 1H, H6), 2.82 (ddd, J = 17.9 Hz, 6.6 Hz, 2.7 Hz, 1H, H5), 3.17 (ddd, J = 17.7 Hz, 11.0 Hz, 6.4 Hz, 1H, H5), 4.29 (bs, OH), 4.41 (m, 1H, H7), 4.64 (d, J = 3.1 Hz, 1H, H8), 7.55 (d, J = 7.9 Hz, 1H, H3), 7.59 (d, J = 7.9 Hz, 1H, H4).

¹³C NMR (100 MHz, CDCl₃): δ 23.8 (t, C5), 25.0 (t, C6), 66.4 (d, C7), 70.6 (d, C8), 117.2 (s, CN), 127.2 (d, C3), 130.6 (s, C2), 136.4 (s, C4a), 137.3 (d, C4), 157.9 (s, C8a).

5.7.5 *cis*-(5R,6S)-5,6-Dihydroxy-5,6,7,8-tetrahydroisoquinoline-1-carbonitrile (**23**)



Diol **13** (50 mg, 0.531 mmol) was filled in a 2-necked 25 mL flask. Dry EtOAc (10 mL) was added and 5 mg Pd catalyst (10 % w/w on charcoal) was added. Hydrogen gas filled in a three layered balloon was purged via syringe and needle and reaction was stirred at room temperature and ambient pressure. Catalytic hydrogenation was carried according to GP10 at 50 PSI overnight. Product formation was monitored by GCMS as both normal and reduced diol have almost same R_f value on the TLC. The catalyst was removed by filtration over Celite[®]. The product was obtained as brownish gummy material.

Yield: 95% (48 mg, 0.252 mmol)

Appearance: brownish gummy material

TLC: 0.60 (EtOAc)

¹H NMR (200 MHz, CDCl₃): δ 1.86 – 2.38 (m, 2H, H7), 2.86-3.07 (m, 1H, H8), 3.07-3.33 (m, 1H, H8), 4.28 (dt, J = 6.3, 2.9 Hz, 1H, H6), 4.67 (d, J = 3.6

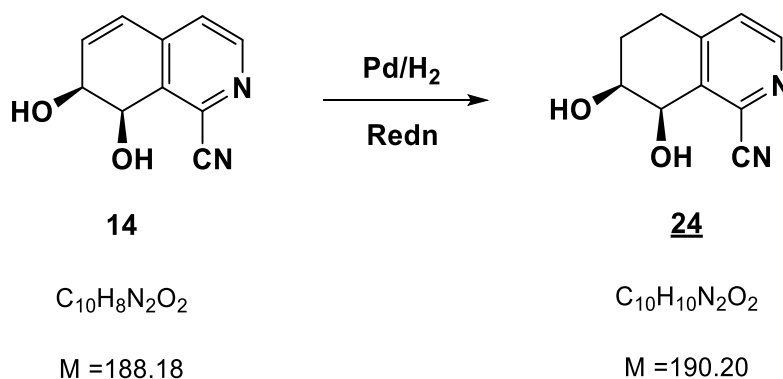
Hz, 1H, H5), 7.72 (d, J = 5.0 Hz, 1H, H4), 8.53 (d, J = 5.0 Hz, 1H, H3).

^{13}C NMR (50 MHz, CDCl_3): δ 22.4 (t, C8), 26.6 (t, C7), 67.2 (d, C5), 69.4 (d, C6), 116.9 (s, CN), 126.9 (d, C4), 132.8 (s, C1), 137.8 (s, C8a), 148.6 (d, C3), 150.5 (s, C4a).

^1H NMR (200 MHz, DMSO) δ 1.87-2.11 (m, 1H, H7), 2.21 (dtd, J = 12.5, 6.2, 3.8 Hz, 1H, H7), 2.91 (ddd, J = 17.8, 6.7, 3.9 Hz, 1H, H8), 3.14 (ddd, J = 16.8, 10.0, 6.4 Hz, 1H, H8), 4.14-4.22 (m, 1H), 4.56-4.67 (m, 1H), 4.76 (bs, OH), 5.45 (bs, OH), 7.81 (d, J = 4.9 Hz, 1H, H4), 8.53 (d, J = 4.9 Hz, 1H, H3).

^{13}C NMR (50 MHz, DMSO): δ 22.6 (t, C8), 26.8 (t, C7), 67.5 (d, C5), 69.7 (d, C6), 117.1 (s, CN), 127.2 (d, C4), 133.1 (s, C1), 138.0 (s, C8a), 148.8 (d, C3), 150.6 (s, C4a).

5.7.6 *cis*- (7S,8R)-7,8-Dihydroxy-5,6,7,8-tetrahydroisoquinoline-1-carbonitrile (**24**)



Diol **14** (50 mg, 0.531 mmol) was filled in a 2-necked 25 mL flask. Dry EtOAc (10 mL) was added and 5 mg Pd catalyst (10 % w/w on charcoal) was added. Hydrogen gas filled in a three layered balloon was purged via syringe and needle and reaction was stirred at room temperature and ambient pressure. Catalytic hydrogenation was carried according to GP10 at 50 PSI overnight. Product formation was monitored by GCMS as both normal and reduced diol have almost same R_f value on the TLC. The catalyst was removed by filtration over Celite[®]. The product was obtained as brownish sticky solid.

Yield: 83% (42 mg 0.221 mmol)

Appearance: yellowish brown solid

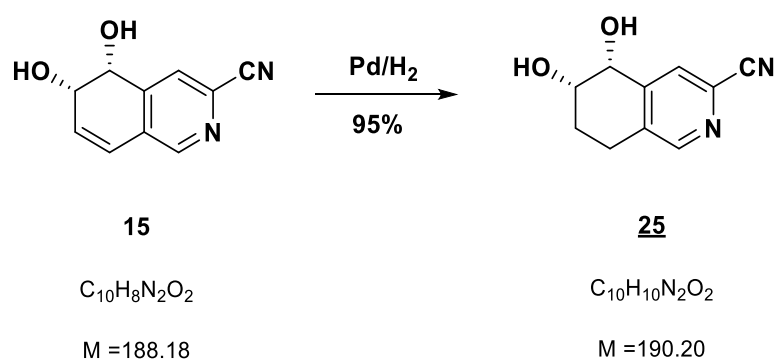
m.p.: 119-122 °C

TLC: 0.50 (EtOAc)

¹H NMR (200 MHz, CDCl₃): δ 1.76 – 2.20 (m, 2H, H6), 2.67 – 3.09 (m, 2H, H5), 3.97 (dt, J = 10.7, 3.6 Hz, 1H, H7), 5.03 (d, J = 3.7 Hz, 1H, H8), 7.23 (s, 1H, H4), 8.45 (d, J = 5.0 Hz, 1H, H3).

¹³C NMR (50 MHz, CDCl₃): δ 24.6 (t, C6), 27.5 (t, C5), 66.2 (d, C8), 68.8 (d, C7), 116.4 (s, CN), 126.9 (d, C4), 135.9 (s, C1), 137.6 (s, C8a), 147.9 (d, C3), 149.4 (s, C4a).

5.7.7 (+)-*cis*-(5R,6S)-5,6-Dihydroxy-5,6,7,8-tetrahydroisoquinoline-3-carbonitrile (**25**)



Diol **15** (100 mg, 0.531 mmol) was filled in the 250 mL Parr hydrogen flask. Dry EtOAc (70 mL) was added and 35 mg Pd catalyst (10 % w/w on charcoal) was added. Catalytic hydrogenation was carried according to GP10 at 50 PSI overnight. The catalyst was removed by filtration over Celite[®]. Then, the reaction solution was passed over a small column of silica and the solvent was evaporated. *cis*-5,6-dihydroxy-5,6,7,8-tetrahydroisoquinoline-3-carbonitrile **25** was purified by passing over small column of silica and evaporating the solvent.

Yield: 92% (93 mg, 0.489 mmol)

Appearance: Beige solid

Elemental Analysis: Calc.: C 63.15%, H 5.30%, N 14.73%

Found: C 62.86%, H 5.33%, N 14.38%

m.p.: 131-132°C (EtOAc: hexane)

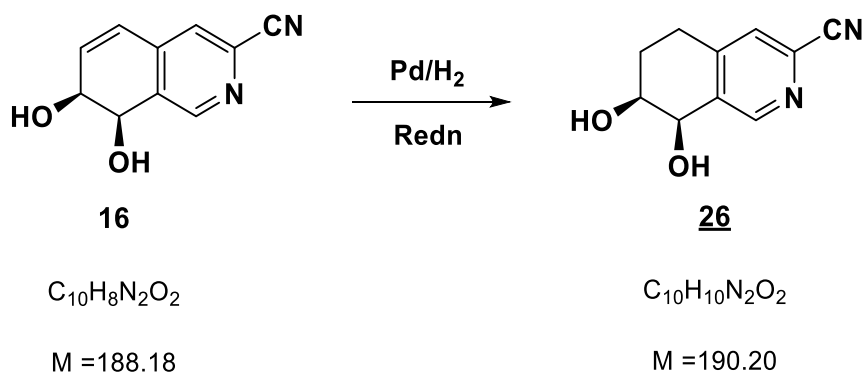
TLC: 0.50 (EtOAc)

$\alpha_D^{20} =$ +44 (C= 0.99, EtOAc)

^1H NMR (200 MHz, DMSO): δ 1.84-2.04 (m, 2H, H7), 2.72 (dt, J = 18.0, 5.5 Hz, 1H, H8), 2.84-2.96 (m, 1H, H8), 3.98 (dt, J = 5.9, 2.9 Hz, 1H, H6), 4.50 (d, J = 2.9, 1H, H5), 7.90 (s, 1H, H4), 8.46 (s, 1H, H1).

^{13}C NMR (50MHz, DMSO): δ 22.1 (t, C7), 26.1 (t, C8), 66.7 (d, C5), 68.4 (d, C6), 118.0 (s, CN), 127.8 (d, C4), 129.7 (s, C3), 137.4 (s, C8a), 149.8 (s, C4a), 150.9 (d, C1).

5.7.8 (+)-*cis*-(7S,8R)-7,8-Dihydroxy-5,6,7,8-tetrahydroisoquinoline-3-carbonitrile (**26**)



Since *cis*-(7S,8R)-7,8-dihydroxy-7,8-dihydroisoquinoline-3-carbonitrile **16** could not be separated from major *cis*-(5R,6S)-5,6-dihydroxy-5,6-dihydroisoquinoline-3-carbonitrile **15**, it was reduced as a mixture. A 100 mg (0.531 mmol) mixture of diols **15** and **16** containing 20% (20 mg, 0.106 mmol) of diol **16** was reduced according to GP10. Mixture of reduced diols **25** and **26** was also not separable via column chromatography. Yield of minor reduced diol is reported from mixture.

Yield: *94% (19 mg, 0.100 mmol) calculated from mixture

Appearance: Yellow gummy material, not isolated

m.p.: not isolated

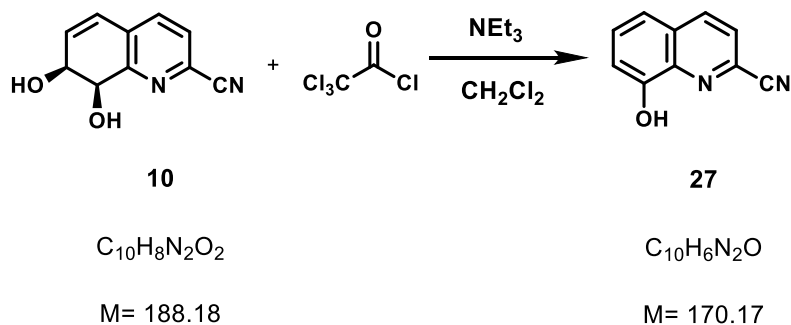
TLC: 0.60 (EtOAc)

$\alpha_D^{20} =$ not isolated

^1H NMR (200 MHz, CDCl_3)*: δ 1.86 – 2.06 (m, 1H, H6), 2.12 – 2.33 (m, 1H, H6), 2.78 (dq, $J = 17.4, 6.2, 5.6$ Hz, 1H, H5), 3.07 (td, $J = 11.1, 9.8, 5.3$ Hz, 1H, H5), 4.15 – 4.23 (m, 1H, H7), 4.80 (d, $J = 3.3$ Hz, 1H, H8), 7.45 (s, 1H, H4), 8.79 (s, 1H, H1).

5.8 Proof of absolute configuration

5.8.1 8-Hydroxy quinolone-2-carbonitrile (**27**)



In a test reaction (7S,8R)-7,8-dihydroxy-7,8-dihydroquinoline-2-carbonitrile (**10**) (50 mg, 0.266 mmol) was dissolved in 5 mL of pyridine at room temperature under N_2 atmosphere. Trichloroacetyl chloride (121 mg, 0.665 mmol, 2.5 eq) was added under N_2 . The reaction was stirred overnight. NMR analysis of crude showed formation of 8-hydroxyquinoline-2-carbonitrile (**27**) instead and no ester formation was observed.²²⁰

Yield: 61% (28 mg, 0.162 mmol)

Appearance: yellow

m.p.: 134-136 °C (lit 134.5-135)²²¹

TLC: 0.50 (EtOAc: hexane, 1: 5)

1H NMR (200 MHz, $CDCl_3$): δ 7.30 (dd, $J = 7.6, 0.9$ Hz, 1H), 7.41 (m, 1H), 7.64 (t, $J = 8.0$ Hz, 1H), 7.72 (d, $J = 8.5$ Hz, 1H), 8.31 (d, $J = 8.5$ Hz, 1H).

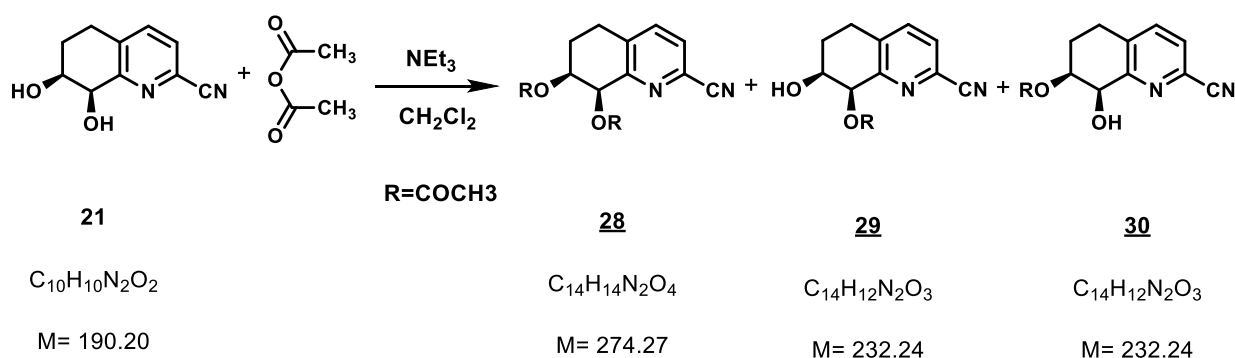
^{13}C NMR (50 MHz, $CDCl_3$): δ 112.2, 118.1, 118.4, 124.1, 129.2, 131.0, 131.4, 137.7, 138.5, 152.4.

1H NMR (200 MHz, DMSO): δ 7.29 (d, $J = 2.4$ Hz, 1H), 7.35 (dd, $J = 8.9, 2.4$ Hz, 1H), 7.77 (d, $J = 8.3$ Hz, 1H), 7.97 (d, $J = 8.9$ Hz, 1H), 8.48 (d, $J = 8.3$ Hz, 1H).

²²⁰ [220] A. V. R. Rao, S. P. Chavan and L. Sivadasan, *Tetrahedron* **1986**, 42, 5065-5071.

²²¹ [221] W. D. Shrader, J. Celebuski, S. J. Kline and D. Johnson, *Tetrahedron Letters* **1988**, 29, 1351-1354.

5.8.2 (+)-*cis*-(7S,8R)-2-Cyano-5,6,7,8-tetrahydroquinoline-7,8-diyl diacetate (**28**)



cis-(7S,8R)-7,8-Dihydroxy-5,6,7,8-tetrahydroquinoline-2-carbonitrile (**21**) (90 mg, 0.473 mmol) was dissolved in 3 mL of dry DCM and cooled to -10°C under N₂ atmosphere. Then, triethylamine 158 mg (1.56 mmol, 3.3 eq) was added followed by acetic anhydride 106 mg (1.04 mmol, 2.2 eq) in 2 mL of DCM (dropwise via a syringe). The reaction mixture was stirred at -10 °C for 30 minutes and before being warmed to room temperature slowly and stirred for an additional 6 hours at that temperature. After completion of reaction, DCM was evaporated, water was added and the reaction mixture was extracted with EtOAc. The organic layer was washed with brine, dried over Na₂SO₄, solvent was evaporated and product was purified by MPLC using EtOAc and hexane gradient mixture (5:1 to 1:1) as eluent. Three product spots were observed on TLC corresponding to all three possible products (diester **28** and monoesters **29** and **30**). By addition of another equivalent of acetic anhydride and stirring overnight, formation of diester was favored. *cis*-(7S,8R)-2-Cyano-5,6,7,8-tetrahydroquinoline-7,8-diyl diacetate **28** was isolated as colorless solid (88 mg, 0.322 mmol).

Yield: 68% (88 mg, 0.322 mmol)

Appearance: Colorless solid

m.p.: 95-97 °C

TLC: 0.50 (EtOAc: hexane, 1: 1)

α_D^{20} = -86 (c=0.9 EtOAc)

¹H NMR (200 MHz, CDCl₃): δ 2.05 (s, 3H, CH₃), 2.07 – 2.38 (m, 5H, CH₃ + CH₂-6), 3.01 (qt, J = 18.0 Hz, 6.7 Hz, 2H, CH₂-5), 5.43 (dt, J = 8.6 Hz, 3.2

Hz, 1H, H7), 6.18 (d, $J = 3.7$ Hz, 1H, H8), 7.50 – 7.69 (m, 2H, H₂ + H₃).

¹³C NMR (50 MHz, CDCl₃): δ 20.6 (q, CH₃), 20.7 (q, CH₃), 22.6 (t, C6), 24.7 (t, C5), 66.8 (d, C7), 67.0 (d, C8), 117.4 (s, CN), 128.3 (d, C3), 130.5 (s, C2), 137.8 (s, C4a), 138.7 (d, C4), 153.5 (s, C8a), 169.6 (s, CO), 169.7 (s, CO).

¹H NMR (200 MHz, DMSO): δ 1.94 (d, $J = 0.6$ Hz, 3H, CH₃), 1.97 – 2.13 (m, 5H, CH₃ + CH₂-6), 2.97 (q, $J = 6.4$ Hz, 2H, CH₂-5), 5.27 (td, $J = 6.2$ Hz, 3.7 Hz, 1H, H7), 6.07 (d, $J = 3.7$ Hz, 1H, H8), 7.79 – 7.97 (m, 2H, H₂ + H₃).

5.8.3 *cis*-(7S,8R)-2-cyano-7-hydroxy-5,6,7,8-tetrahydroquinolin-8-yl acetate (**29**)

First fraction was diester **28** with 30% yield. Second fraction being acetate ester **29** was isolated as colorless solid 26 mg (0.112 mmol).

Yield: 24% (26 mg, 0.112 mmol)

Appearance: colorless solid

TLC: 0.4 (EtOAc: hexane, 1: 1)

¹H NMR (200 MHz, CDCl₃): δ 2.03 (s, 3H, CH₃), 2.06 – 2.43 (m, 2H, H6), 2.75 – 3.21 (m, 2H, H5), 3.80 (s, OH), 4.81 (d, $J = 3.5$ Hz, 1H, H8), 5.52 (ddd, $J = 6.2$ Hz, 3.5 Hz, 2.4 Hz, 1H, H7), 7.53 – 7.67 (m, 2H, H₂ + H₃).

5.8.4 *cis*-(7S,8R)-2-Cyano-8-hydroxy-5,6,7,8-tetrahydroquinolin-7-yl acetate (**30**)

From the reaction of reduced diol **21** with acetic anhydride as mentioned for monoester **29**, monoester **30** was isolated as most polar fraction with R_f of 0.3. Total isolated yield of all three products combined was about 72%. (7S,8R)-2-cyano-8-hydroxy-5,6,7,8-tetrahydroquinolin-7-yl acetate (**30**) was isolated as colorless solid (20 mg, 0.112 mmol).

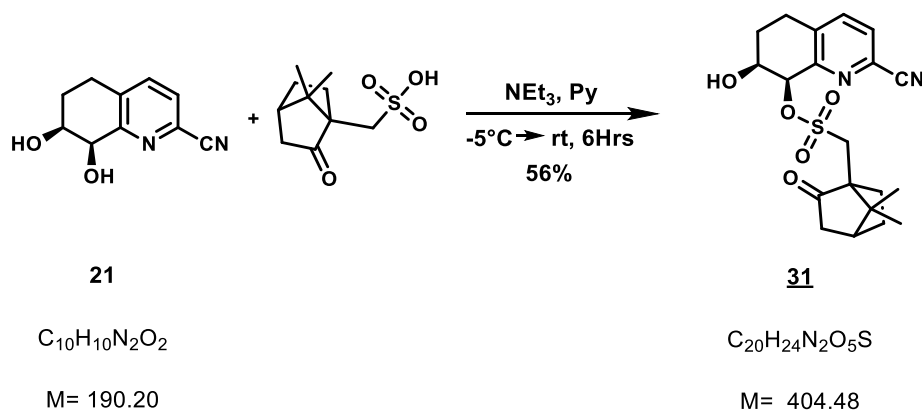
Yield: 18% (20 mg, 0.086 mmol)

Appearance: colorless solid

TLC: 0.3 (EtOAc: hexane, 1: 1)

¹H NMR (200 MHz, CDCl₃): δ 2.12 – 2.42 (m, 5H, CH₃+H6), 2.70 – 3.45 (m, 2H, H5), 3.76 (s, OH), 4.37 (dt, J = 7.2 Hz, 3.0 Hz, 1H, H7), 6.06 (d, J = 3.5 Hz, 1H, H8), 7.50 – 7.69 (m, 2H, H2 + H3).

5.8.5 (7S,8R)-2-Cyano-7-hydroxy-5,6,7,8-tetrahydroquinolin-8-yl (7,7-dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl)methane sulfonate (**31**)



Reduced diol **21** (40 mg, 0.210 mmol) was dissolved in 5 mL of pyridine. Triethylamine (116.89 mg, 1.16 mmol, 5.5 eq) was added to the flask and cooled to -10°C under N₂. Then camphorsulfonyl chloride (116.1 mg, 0.463 mmol, 2.2 eq) was dissolved in 5 mL DCM and added dropwise via a syringe. The reaction mixture was stirred at -10 °C for 30 minutes and before being warmed to room temperature slowly and stirred for an additional 6 hours at that temperature. TLC showed very small formation of product therefore reaction mixture was heated to 60 °C for 2 hours. Pyridine was evaporated under reduced pressure, water was added and reaction mixture was extracted with EtOAc. Organic layer was washed with brine and dried over Na₂SO₄ and product was purified by MPLC using EtOAc and hexane gradient mixture (4:1 to 1:1) as eluent. This monoester **31** was the only product of esterification reaction carried out with reduced diol **21** and was isolated after MPLC brownish solid.

Yield: 56% (48 mg, 0.119 mmol)

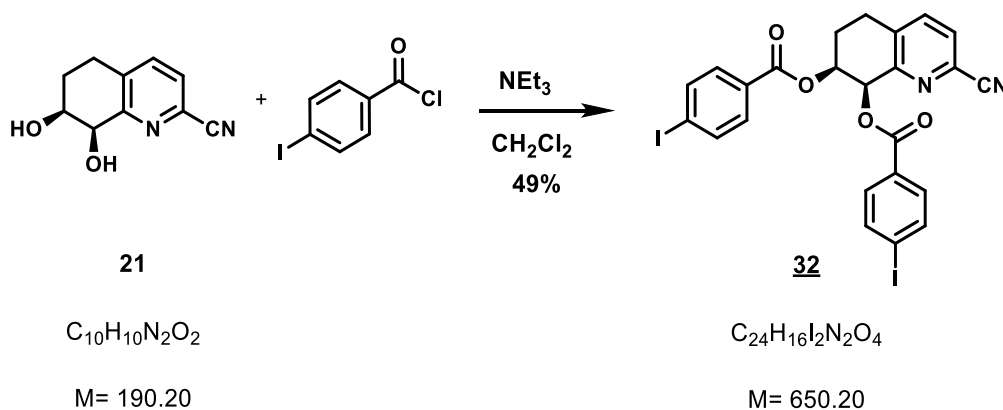
Appearance: beige gummy material

TLC: 0.50 (EtOAc: hexane, 7: 3)

¹H NMR (200 MHz, DMSO): δ 0.79 (s, 3H, CH₃), 1.00 (s, 3H, CH₃), 1.12 – 1.62 (m, 4H, 2xCH₂), 1.81 – 2.43 (m, 5H, CH₂+CH₂-6+CH), 2.90 – 3.08 (m, 2H, CH₂-5), 3.35 (d, J = 15.0 Hz, 1H, CH), 3.63 (d, J = 15.2 Hz, 1H, CH), 4.84 (dd, J = 6.0, 3.3 Hz, 1H, H8), 5.12 (dt, J = 8.0, 3.1 Hz, 1H, H-7), 6.12 (d, J = 6.0 Hz, 1H, OH), 7.84 (d, J = 8.0 Hz, 1H, H3), 7.91 (d, J = 7.9 Hz, 1H, H4).

¹³C NMR (50 MHz, CDCl₃): δ 19.1 (t, CH₃), 19.2 (t, CH₃), 23.6 (t, C6), 24.4 (t, C5), 26.2 (t, CH₂), 41.9 (t, CH₂), 42.1 (d, CH), 47.1 (t, CH₂), 47.7 (t, CH₂), 57.3 (s, C), 68.5 (d, C7), 80.0 (d, C8), 94.2 (t), 117.5 (s, CN), 127.6 (d, C3), 130.0 (s, C2), 136.2 (s, C4a), 138.0 (d, C4), 157.8 (s, C8a), 213.8 (s, CO).

5.8.6 (+)-*cis*-(7S,8R)-2-Cyano-5,6,7,8-tetrahydroquinoline-7,8-diyl bis(4-iodobenzoate) (**32**)



Reduced diol **21** (100 mg, 0.53 mmol) was dissolved in 5 mL of dry DCM and cooled to -10°C under N₂ atmosphere. Then triethylamine (175 mg, 1.73 mmol, 3.3 eq) was added to the reaction. 4-iodobenzoyl chloride (294 mg, 1.10 mmol, 2.1 eq) dissolved in 5 mL DCM was added to the reaction dropwise via a syringe. The reaction was stirred at -10 °C for 30 minutes before being warmed to room temperature slowly and stirred for an additional 6 hours at that temperature. TLC showed formation of two products, less polar product being diester **32** of reduced diol **21**. DCM was evaporated under reduced pressure and water was added and reaction mixture was extracted with EtOAc. Organic layer was washed with brine and dried over Na₂SO₄ and product was purified by MPLC using EtOAc and hexane as

solvents (silica gel, gradient chromatography, EtOAc: PE, 0-20% EtOAc 20 min, 20%-50% EtOAc 20 min, 50%-100% EtOAc 30 min).

Yield: 49% (167 mg, 0.26 mmol)

Appearance: Colorless solid

Elemental Analysis: Calc.: C 44.33%, H 2.48%, N 4.31%, I 39.04%, O 9.84%

Found: C 44.17%, H 2.43%, N 4.28%

m.p.: 95-97 °C (EtOAc: hexane)

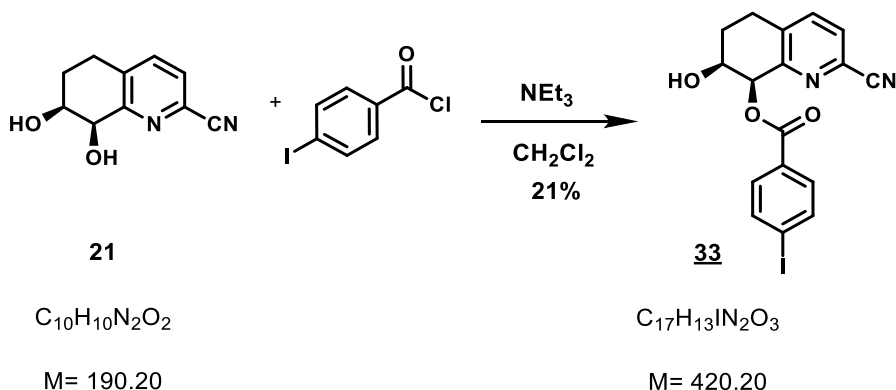
TLC: 0.60 (EtOAc: PE; 8: 2)

α_D^{20} +74 (c= 0.530, CHCl₃)

¹H NMR (400 MHz, CDCl₃): δ 2.29 (dtd, J = 8.7 Hz, 6.3 Hz, 2.5 Hz, 1H, H6), 2.46 (td, J = 13.8 Hz, 6.9 Hz, 1H, H6), 3.07 (dt, J = 18.1 Hz, 6.9 Hz, 1H, H5), 3.22 (dt, J = 18.0 Hz, 6.2 Hz, 1H, H5), 5.73 (dt, J = 9.0 Hz, 3.0 Hz, 1H, H7), 6.54 (d, 1H, J = 3.5 Hz, 1H, H8), 7.55 (d, J = 8.5 Hz, 2H, aryl H), 7.63 (d, J = 7.9 Hz, 1H, H4), 7.68 (d, J = 8.6 Hz, 2H, aryl H), 7.73 (d, J = 8.5 Hz, 1H, H3), 7.78 (d, J = 8.5, 2H, aryl H).

¹³C NMR (CDCl₃, 100MHz): δ 23.8 (t, C6), 25.6 (t, C5), 70.2 (d, C7), 70.3 (d, C8), 101.4 (s, C-I), 101.5 (s, C-I), 117.1 (s, CN), 128.1 (d, C3), 129.2 (s, C-CO), 129.3 (s, C-CO), 131.2 (s, C2), 131.5 (d, aryl CH), 132.6 (d, aryl CH), 136.7 (s, C4a), 137.9 (d, C4), 138.0 (d, aryl CH), 153.9 (s, C8a), 165.3 (s, CO), 165.3 (s, CO).

5.8.7 (+)-*cis*-(7*S*,8*R*)-2-Cyano-7-hydroxy-5,6,7,8-tetrahydroquinolin-8-yl 4-iodobenzoate (**33**)



The reaction of reduced diol **21** with 4-iodobenzoyl chloride at 8-position of diol produced monoester **33**. Monoester formation at 7-position was less favored and only traces of product were observed. Monoester **33** was the minor product of esterification reaction and was isolated as more polar fraction after MPLC.

Yield: 21% (47 mg, 0.112 mmol)

Appearance: Colorless crystals

Elemental Analysis: Calc.: C 48.59%, H 3.12%, N 6.67%, I 30.20% O 11.42%

Found: C 48.92%, H 3.01%, N 6.43%

m.p.: 169-171 °C (EtOAc: hexane)

TLC: 0.60 (EtOAc : PE; 1: 1)

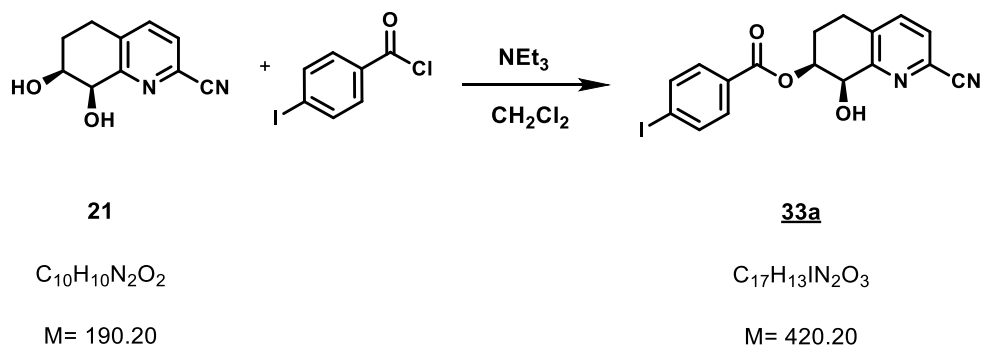
$\alpha_D^{20} =$ +79 (c=0.69, CHCl_3), +97 (c=0.78, EtOAc)

^1H NMR (400 MHz, CDCl_3): δ 2.10 (ddd, J = 14.1 Hz, 8.3 Hz, 2.1 Hz, 1H, H6), 2.25 (td, J = 13.7 Hz, 6.1 Hz, 1H, H6), 2.50 (s, OH), 2.89 (dt, J = 17.9 Hz, 6.1 Hz, 1H, H5), 3.22 (m, 1H, H5), 4.47 (dt, J = 6.0 Hz, 2.6 Hz, 1H, H7), 6.28 (d, J = 3.3 Hz, 1H, H8), 7.56 (d, J = 7.9 Hz, 1H, H3), 7.63 (d, J = 7.9 Hz, 1H, H4), 7.77 (d, J = 8.5 Hz, 2H, aryl CH), 7.81 (d, J = 8.5 Hz, 2H, aryl CH).

^{13}C NMR (100MHz, CDCl_3): δ 24.9 (t, C6), 25.8 (t, C5), 67.7 (d, C7), 73.1 (d, C8), 101.5 (s, Ar-C-I), 117.3 (s, CN), 127.8 (d, C3), 129.3 (s, C2), 131.6

(d, Ar-CH), 132.1 (s, Ar-C-CO), 137.4 (s, C4a), 137.6 (d, Ar-CH), 138.0 (d, C4), 154.3 (s, C8a), 166.2 (s, CO).

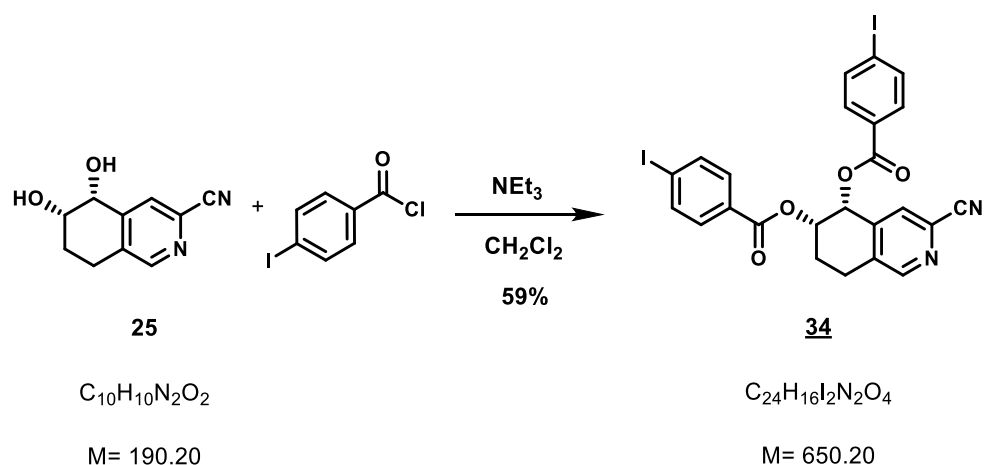
5.8.8 *cis*-(7*S*,8*R*)-2-Cyano-8-hydroxy-5,6,7,8-tetrahydroquinolin-7-yl 4-iodobenzoate (**33a**)



The reaction of reduced diol **21** with 4-iodobenzoyl chloride at 8-position of diol produced monoester **33**. Monoester formation at 7-position was less favored and only traces of product were observed. Monoester **33a** was isolated as most polar fraction and about 8 mg of product were recovered.

¹H NMR (200 MHz, DMSO): δ 1.94 – 2.11 (m, 2H, CH₂-6), 2.79 – 2.99 (m, 1H, H5), 3.00 – 3.19 (m, 1H, H5), 4.24 (dt, $J = 9.5$ Hz, 5.0 Hz, 1H, H7), 5.40 (d, $J = 4.3$ Hz, OH), 6.17 (d, $J = 3.4$ Hz, 1H, H8), 7.78 (d, $J = 8.4$ Hz, 2H, H3+H4), 7.86 – 7.99 (m, 4H, aryl-H).

¹³C NMR (50 MHz, DMSO): δ 24.8 (t, C6), 25.7 (t, C5), 65.9 (d, C8), 72.9 (d, C7), 101.9 (s, aryl-C-I), 117.6 (s, CN), 128.0 (d, C3), 129.4 (s, C4a), 130.2 (s, aryl C-CO), 131.3 (d, aryl-CH, 2C), 137.7 (d, aryl-CH, 2C), 138.3 (d, C4), 138.30 (s, C8a), 164.9 (s, CO).

5.8.9 (+)-*cis*-(5R,6S)-3-Cyano-5,6,7,8-tetrahydroisoquinoline-5,6-diyl bis(4-iodobenzoate) (34)

cis-(5R,6S)-5,6-Dihydroxy-5,6,7,8-tetrahydroisoquinoline-3-carbonitrile (**25**) (100 mg, 0.53 mmol) was reacted with 2.1 equivalents of 4-iodobenzoyl chloride in DCM in presence of 3.3 equivalents of triethylamine using identical conditions described for reduced quinoline diol **25**. Products were purified by MPLC using EtOAc and hexane as solvents (silica gel, gradient chromatography, EtOAc: PE, 0-20% EtOAc 20 min, 20%-50% EtOAc 20 min, 50%-100% EtOAc 30 min). After column chromatography diester **34** of diol **25** was isolated as colorless solid.

Yield: 51% (175 mg, 0.27 mmol)

Appearance: Colorless to beige solid

Elemental Analysis: Calc.: C 44.33%, H 2.48%, N 4.31%, I 39.04%, O 9.84%

Found: C 44.28%, H 2.24%, N 4.19%

m.p.: 95-97 °C (EtOAc: hexane)

TLC: 0.70 (EtOAc: hexane, 2: 8)

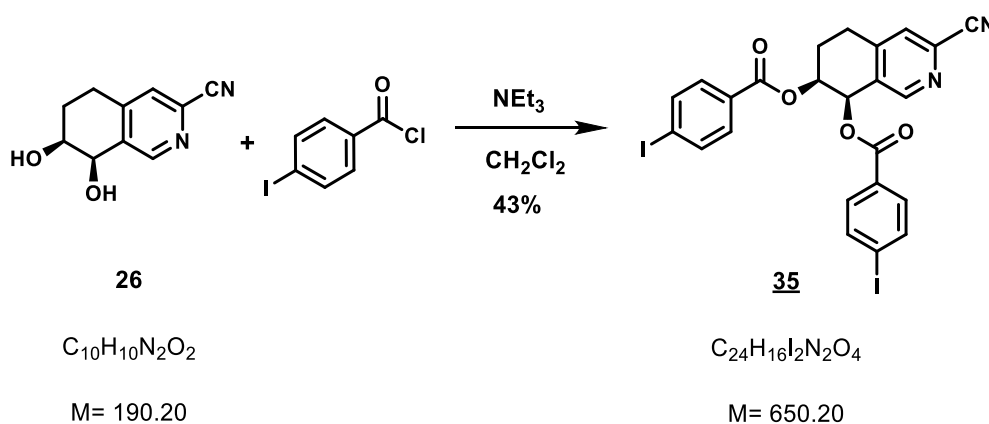
$\alpha_D^{20} =$ +139 (c= 1.035, CHCl₃)

¹H NMR (400 MHz, CDCl₃): δ 2.33 (ddd, J = 14.3 Hz, 8.5 Hz, 2.3 Hz, 1H, H7), 2.53 (td, J = 13.9 Hz, 6.0 Hz, 1H, H7), 3.10 (dt, J = 18.3 Hz, 6.2 Hz, 1H, H8), 3.24 (m, 1H, H8), 5.81 (dt, J = 6.3 Hz, 2.8 Hz, 1H, H6), 6.39 (d, J = 3.5 GHz, 1H, H5), 7.53 (d, J = 8.6 Hz, 2H, aryl

CH), 7.66 (s, 1H, H4), 7.69 (dd, $J = 4.7$ Hz, 4.0 Hz, 2H, aryl CH), 7.76 (d, $J = 8.7$ GHz, 2H, aryl CH), 7.83 (d, $J = 8.7$ Hz, 2H, aryl CH), 8.65 (s, 1H, H1).

^{13}C NMR (100MHz, CDCl_3): δ 23.2 (t, C8), 24.1 (t, C7), 68.6 (d, C5), 68.9 (d, C6), 101.7 (s, CH-I), 102.2 (s, CH-I), 117.2 (s, CN), 127.6 (d, C4), 128.4 (s, C-CO), 129.0 (s, C-CO), 131.1 (aryl CH), 131.4 (aryl CH), 132.2 (s, C3), 136.3 (s, C8a), 138.1 (d, aryl CH), 138.3 (d, aryl CH), 142.9 (s, C4a), 151.9 (d, C1), 165.3 (s, CO), 165.5 (s, CO).

5.8.10 (-)-*cis*-(7S,8R)-3-Cyano-5,6,7,8-tetrahydroisoquinoline-7,8-diyl bis(4-iodobenzoate) (**35**)



As tetrahydroisoquinoline diol **26** was not isolated from major diol **25**, they were reduced as mixture. A 100 mg (0.53 mmol) mixture (85:15) of reduced diols **25** and **26** containing 15 mg (0.08 mmol) of *cis*-(7S,8R)-7,8-dihydroxy-5,6,7,8-tetrahydroisoquinoline-3-carbonitrile (**26**) was reacted with 4-iodobenzoyl chloride using identical conditions described for reduced quinoline diol **26**. After column chromatography diester **35** of minor diol **26** was separated as first least polar fraction.

Yield: 6.4% (22 mg, 0.034 mmol), 43% based on 0.079 mmol of **26**

Appearance: Colorless to beige solid

Elemental Analysis: Calc.: C 44.33%, H 2.48%, N 4.31%, I 39.04%, O 9.84%

Found: C 44.89%, H 2.50%, N 4.16%

m.p.: 95-97 °C (EtOAc: hexane)

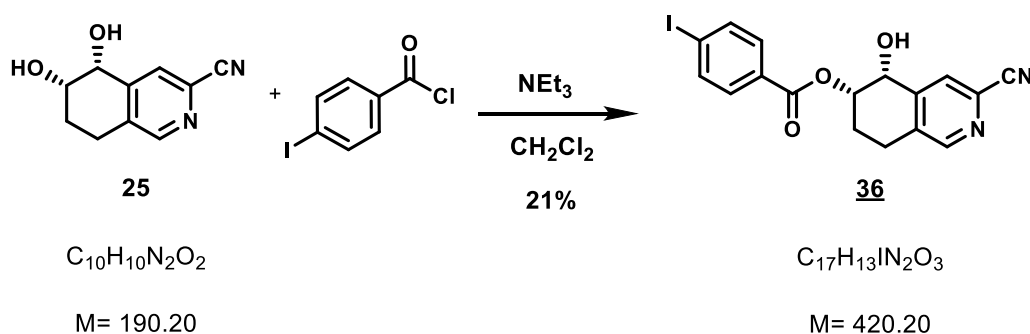
TLC: 0.70 (EtOAc: hexane, 2: 8)

$\alpha_D^{20} =$ -20 (C= 1.08, CHCl₃)

¹H NMR (200 MHz, CDCl₃): δ 2.30 (dt, J = 10.2 Hz, 6.6 Hz, 1H, H6), 2.45 (dt, J = 9.1 Hz, 7.0 Hz, 1H, H6), 3.13 (m, 2H, H5), 5.68 (dt, J = 9.1 Hz, 3.2 Hz, 1H, H7), 6.53 (d, J = 3.4 Hz, 1H, H8), 7.53 (d, J = 8.5 Hz, 2H, aryl CH), 7.58 (s, 1H, H4), 7.67 (d, J = 8.6 Hz, 2H, aryl CH), 7.74 (d, J = 8.6 Hz, 2H, aryl CH), 7.80 (d, J = 8.6 Hz, 2H, aryl CH), 8.70 (s, 1H, H1).

¹³C NMR (50 MHz, CDCl₃): δ 23.3 (t, C6), 26.0 (t, C5), 67.8 (d, C8), 69.3 (d, C7), 101.7 (s, CH-I), 102.0 (s, CH-I), 117.1 (s, CN), 128.3 (d, C4), 128.4 (s, C-CO), 128.7 (s, C-CO), 129.0 (s, C3), 131.1 (d, aryl CH), 131.3 (d, aryl CH), 132.8 (s, C4a), 133.7 (s), 137.8 (d, aryl CH), 138.1 (d, aryl CH), 138.2 (d, aryl CH), 147.4 (s, C8a), 152.3 (d, C1), 165.3 (s, CO), 165.4 (s, CO).

5.8.11 (+)-*cis*-(5R,6S)-3-Cyano-5-hydroxy-5,6,7,8-tetrahydroisoquinoline-6-yl-4-iodobenzoate (**36**)



cis-(5R,6S)-5,6-dihydroxy-5,6,7,8-tetrahydroisoquinoline-3-carbonitrile (**25**) (100 mg, 0.525 mmol) was reacted with 2.1 equivalents of 4-iodobenzoyl chloride in DCM in presence of 3.3 equivalents of triethylamine using identical conditions described for reduced quinoline diol **25**. After column chromatography monoester **36** of major diol **25** was isolated as colorless solid.

Yield: 33 % (73 mg, 0.173 mmol)

Appearance: colorless solid

Elemental Analysis: Calc.: C 48.59%, H 3.12%, N 6.67%, I 30.20% O 11.42%

Found: C 48.36%, H 2.80%, N 6.49%

m.p.: 153-154°C (EtOAc-hexane)

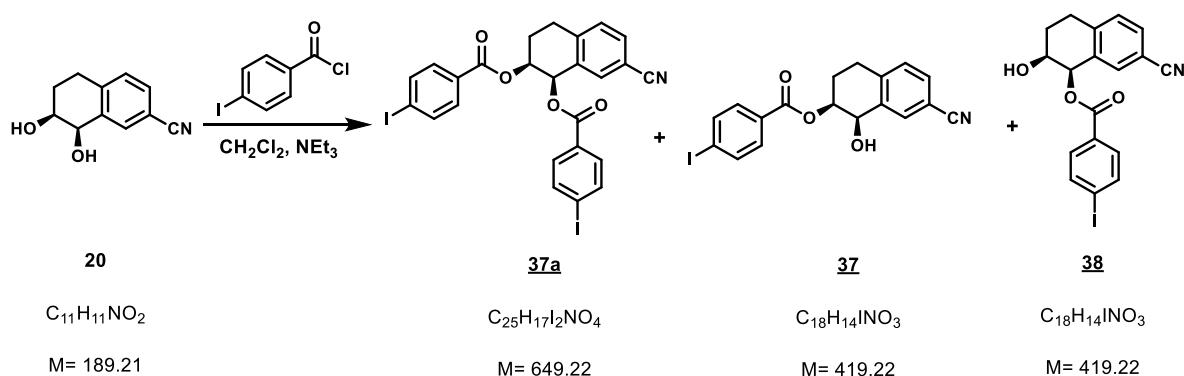
TLC: 0.60 (EtOAc: hexane, 8: 2)

$\alpha_D^{20} =$ +113 (c=0.610, CHCl₃), +136 (c=0.77, EtOAc)

¹H NMR (200 MHz, CDCl₃): δ 2.16 (m, 1H, H7), 2.44 (m, 1H, H7), 3.11 (m, 3H, H8 + OH), 4.93 (d, J = 3.4, 1H, H5), 5.60 (m, 1H, H6), 7.54 (d, J = 8.6, 2H, aryl CH), 7.72 (d, J = 6.8, 2H, aryl CH), 7.89 (s, 1H, H4), 8.48 (s, 1H, H1).

¹³C NMR (50 MHz, CDCl₃): δ 22.5 (t, C7), 23.9 (t, C8), 67.9 (d, C6), 71.3(d, C5), 101.5 (s, CH-I), 117.3 (s, CN), 127.5 (d, C4), 128.8 (s, C-CO), 130.9 (d, aryl CH), 131.7 (s, C3), 135.4 (s, C4a), 137.9 (d, aryl CH), 146.6 (s, C8a), 151.1 (d, C1), 166.0 (s, CO).

5.8.12 *cis*-(1R,2S)-7-Cyano-1,2,3,4-tetrahydronaphthalene-1,2-diyl bis(4-iodobenzoate) (**37a**)



cis-7,8-dihydroxy-5,6,7,8-tetrahydronaphthalene-2-carbonitrile (**20**) (568 mg, 3 mmol) was dissolved in 10 mL of dry DCM and cooled to -10°C under N₂ atmosphere. Then triethylamine (1002 mg, 9.9 mmol, 3.3 eq) was added to the reaction mixture. 4-iodobenzoyl chloride (1679 mg, 6.3 mmol, 2.1 eq) was dissolved in 15 mL DCM and added dropwise via syringe. The reaction mixture was stirred at -10 °C for 30 minutes before being warmed to

room temperature slowly and stirred for an additional 6 hours at that temperature. Three product spots were observed on TLC corresponding to all three possible products (diester **37a** and monoesters **37** and **38**). DCM was evaporated under reduced pressure and water was added and reaction mixture was extracted with EtOAc. Organic layer was washed with brine and dried over Na₂SO₄ and product was purified by MPLC using EtOAc and hexane as solvents (silica gel, gradient chromatography, EtOAc: PE, 0-20% EtOAc 20 min, 20%-50% EtOAc 20 min, 50% EtOAc 30 min).

Yield: 9 % (173 mg, 0.27 mmol)

Appearance: brown gummy material

TLC: 0.6 (EtOAc: hexane, 8: 2)

¹H NMR (200 MHz, CDCl₃): δ 2.15 – 2.35 (m, 1H, H3), 2.36 – 2.59 (m, 1H, H3), 2.95 – 3.33 (m, 2H, H4), 5.63 (dt, J = 9.6 Hz, 3.4 Hz, 1H, H2), 6.45 (d, J = 3.4 Hz, 1H, H1), 7.33 (d, J = 8.0 Hz, 1H, H5), 7.48 – 7.61 (m, 3H, H6+Ar-H), 7.64 – 7.82 (m, 7H, H8+Ar-H).

¹³C NMR (101 MHz, CDCl₃): δ 23.5 (t, C3), 26.9 (t, C4), 69.4 (d, C1), 70.0 (d, C2), 101.2 (s, Ar-C-I), 102.0 (s, Ar-C-I), 110.7 (s, C7), 118.4 (s, CN), 128.9 (s, Ar-CO-C), 129.1 (s, Ar-CO-C), 129.8 (d, C5), 131 (d, Ar-C), 131.1 (d, Ar-C), 132 (d, C6), 133.5 (d, C8), 134 (s, C8a), 137.8 (d, aryl CH), 138.0 (d, aryl CH), 142.0 (s, C4a), 165.2 (s, CO), 165.4 (s, CO).

5.8.13 (+)-*cis*-(1R,2S)-7-cyano-1-hydroxy-1,2,3,4-tetrahydronaphthalen-2-yl 4-iodobenzoate (**37**)

Yield: 26 % (327 mg, 0.780 mmol)

Appearance: brown gummy material

TLC: 0.4 (EtOAc: hexane, 8: 2)

¹H NMR (200 MHz, CDCl₃): δ 1.91 – 2.13 (m, 1H, H3), 2.33 (dt, J = 14.4 Hz, 7.1 Hz, 1H, H3), 2.73 – 3.10 (m, 2H, H4), 4.88 (d, J = 3.4 Hz, 1H, H1), 5.42 (dt, J = 8.2 Hz, 3.0 Hz, 1H, H2), 7.12 – 7.24 (m, 1H,

H5), 7.44 (dd, $J = 8.0, 1.7$ Hz, 1H, H3/H1), 7.50 – 7.62 (m, 2H, H1/H3 + Ar-H), 7.64 – 7.80 (m, 3H, Ar-H).

^{13}C NMR (101 MHz, CDCl_3): δ 23.4 (t, C3), 26.3 (t, C4), 68.3 (d, C1), 72.5 (d, C2), 101.3 (s, Ar-C-I), 110.5 (s, C7), 118.7 (s, CN), 128.1 (s, Ar-CO-C), 129.3 (d, C5/C6), 131.2 (d, Ar-C), 132 (d, C6/C5), 133.1 (d, C8), 137.9 (s, C8a), 138 (d, aryl CH), 141.4 (s, C4a), 165.9 (s, CO).

5.8.14 (+)-*cis*-(1R,2S)-7-cyano-2-hydroxy-1,2,3,4-tetrahydronaphthalen-1-yl 4-iodobenzoate (38)

Yield: 21% (265 mg, 0.632 mmol)

Appearance: yellowish brown gummy material

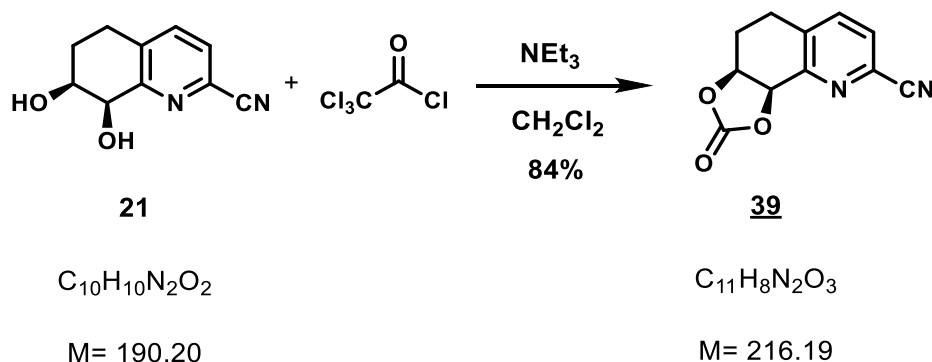
TLC: 0.45 (EtOAc: hexane, 8: 2)

^1H NMR (200 MHz, CDCl_3): δ 2.01 – 2.37 (m, 2H, H3), 2.91 (dt, $J = 17.8$ Hz, 7.0 Hz, 1H, H4), 3.19 (dt, $J = 18.0$ Hz, 6.2 Hz, 1H, H4), 4.34 (dt, $J = 8.7$ Hz, 3.3 Hz, 1H, H2), 6.24 (d, $J = 3.4$ Hz, 1H, H1), 7.31 (m, 1H, H5), 7.53 (dd, $J = 8.0$ Hz, 1.6 Hz, 1H, H6), 7.66 (s, 1H, H8), 7.71 – 7.86 (m, 4H, Ar-H).

^{13}C NMR (50 MHz, CDCl_3): δ 26.2 (t, C3), 26.4 (t, C4), 67.5 (d, C1), 72.3 (d, C2), 101.7 (s, Ar-C-I), 110.5 (s, C7), 118.7 (s, CN), 128.1 (s, Ar-CO-C), 130.2 (d, C5/C6), 131.7 (d, Ar-C), 132.7 (d, C6/C5), 133.9 (d, C8), 137.6 (s, C8a), 138 (d, aryl CH), 143 (s, C4a), 165.9 (s, CO).

5.9 Cyclic carbonate synthesis

5.9.1 (+)-*cis*-(3a*S*,9b*R*)-2-Oxo-3a,9b-tetrahydro-[1,3]dioxolo[4,5-*h*]quinoline-8-carbonitrile (**39**)



The reaction was performed according to GP8. Reduced diol **21** (30 mg, 0.16 mmol) was dissolved in 5 mL of dry DCM. The reaction was cooled in ice bath to $-10\text{ }^\circ\text{C}$ under N_2 atmosphere. First, triethylamine (53 mg, 0.52 mmol, 3.3 eq) was added to the reaction and then freshly distilled trichloroacetyl chloride (63 mg, 0.35 mmol, 2.2 eq) diluted with 2 mL DCM was added dropwise. The reaction was stirred at $-10\text{ }^\circ\text{C}$ for 30 minutes and then slowly warmed to room temperature and stirred for 6 hours at that temperature. After completion of reaction, DCM was evaporated under reduced pressure, water was added and the reaction mixture was extracted three times with 20 mL EtOAc. The organic layer was washed with brine and dried over Na_2SO_4 and the solvent was evaporated. Cyclic carbonate **39** was purified by MPLC using EtOAc and hexane mixture (1:1) as eluent.

Yield: 84% (29 mg, 0.13 mmol)

Appearance: Colorless crystals

Elemental Analysis: Calc.: C 61.11%, H 3.73%, N 12.96%

Found: C 60.88%, H 3.60%, N 12.94%

m.p.: 239-241 $^\circ\text{C}$ (EtOAc: hexane)

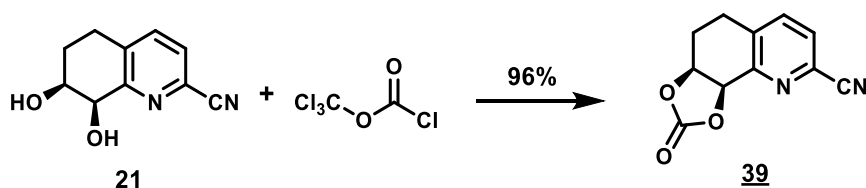
TLC: 0.50 (EtOAc: PE ; 8: 2)

$\alpha_{\text{D}}^{20} =$ +60 (c=0.45, EtOAc)

¹H NMR (200 MHz, DMSO): δ 1.89 – 2.30 (m, 2H, H6), 2.77 – 3.03 (m, 2H, H5), 5.41 (dt, $J = 8.1$ Hz, 4.2 Hz, 1H, H7), 5.82 (d, $J = 8.0$ Hz, 1H, H8), 7.92 – 8.12 (m, 2H, H3+H4).

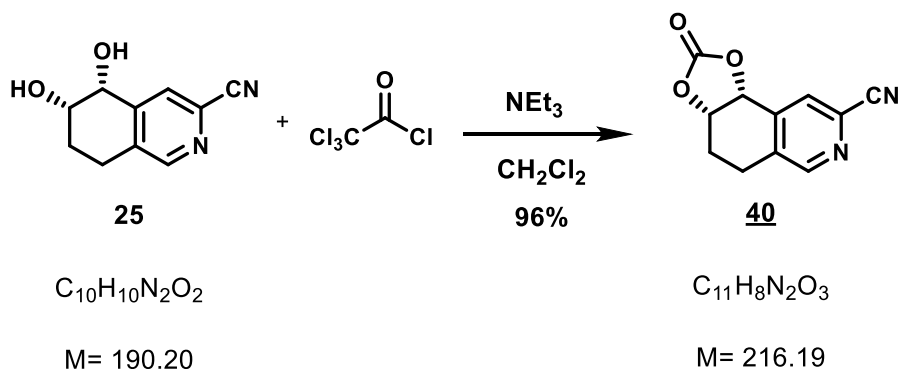
¹³C NMR (50 MHz, DMSO): δ 22.5 (t, C6), 25.1 (t, C5), 75.1 (d, C8), 75.4(d, C7), 117.1 (s, CN), 129.0 (d, C3), 131.0 (s, C2), 138.8 (d, C4), 139.1 (s, C4a), 151.7 (s, C8a), 154.0 (s, CO).

5.9.2 Alternate procedure for preparation of **39**



The reaction was performed according to GP8. Reduced diol **21** (30 mg, 0.16 mmol) was dissolved in 5 mL of dry DCM. The reaction was cooled in an ice bath to -10 °C under N₂ atmosphere. Triethylamine (88 mg, 0.87 mmol, 5.5 eq) was added to the reaction under N₂. Then, trichloromethyl chloroformate (69 mg (0.35 mmol, 2.2 eq) mixed with 3 mL DCM was slowly added dropwise via needle and syringe. Purification of product after MPLC using PE:EtOAc (1:1) showed cyclic carbonate **39** (33 mg, 0.15 mmol) with 96% yield.

5.9.3 (+)-*cis*-(3a*S*,9b*R*)-2-oxo-3a,4,5,9b-tetrahydro-[1,3]dioxolo[4,5-*f*]isoquinoline-8-carbonitrile (**40**)



Reduced diol **25** (150 mg, 0.789 mmol) was dissolved in 5 mL of dry DCM. The reaction was cooled in ice bath at -10 °C under N₂ atmosphere. First, Triethylamine (263.5 mg, 2.604 mmol, 3.3 eq) was added to the reaction and then freshly distilled trichloroacetyl

chloride (316 mg, 1.74 mmol, 2.2 eq) dissolved in DCM was added dropwise. The purification was performed according to GP11.

Yield: 85% (145 mg, 0.671 mmol)

Appearance: Colorless to beige crystals

m.p.: 133-135 °C

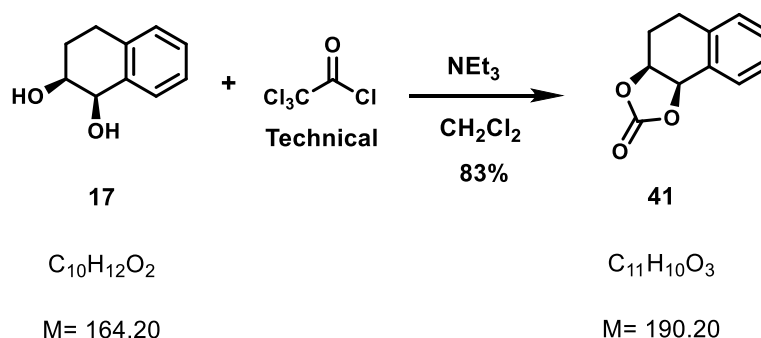
TLC: 0.50 (EtOAc: hexane, 8: 2)

$\alpha_D^{20} =$ +279 (c=0.6, CHCl₃)

¹H NMR (200 MHz, CDCl₃): δ 1.96 – 2.22 (m, 1H, H7), 2.28 – 2.53 (m, 1H, H7), 2.71 – 3.08 (m, 2H, H8), 5.17 – 5.35 (m, 1H, H6), 5.63 (d, J = 7.7 Hz, 1H, H5), 7.76 (s, 1H, H4), 8.63 (s, 1H, H1).

¹³C NMR (50 MHz, CDCl₃): δ 20.8 (t, C8), 25.7 (t, C7), 72.3 (d, C5), 74.3 (d, C6), 116.6 (s, CN), 129.1 (d, C4), 132.8 (s, C3), 136.9 (s, C8a), 139.4 (s, C4a), 151.6 (d, C1), 153.3 (s, CO).

5.9.4 (+)-*cis*-(3a*S*,9b*R*)-3a,4,5,9b-tetrahydronaphtho[1,2-*d*][1,3]dioxol-2-one (41)



Reduced naphthalene diol **17** (248 mg, 1.51 mmol) was reacted with trichloroacetyl chloride (604 mg, 3.32 mmol, 2.2 eq) in presence of triethylamine (504 mg, 4.98 mmol). The purification of cyclic carbonate **41** was performed according to GP11.

Yield: 83% (237 mg, 1.25mmol)

Appearance: colorless solid

m.p.: 96-97°C (EtOAc-hexane) (Lit 96-98 °C)²⁰⁵

$\alpha_D^{20} =$ +219 (c=1.12, CHCl₃), Lit +206.4 (c=1.0, CHCl₃)

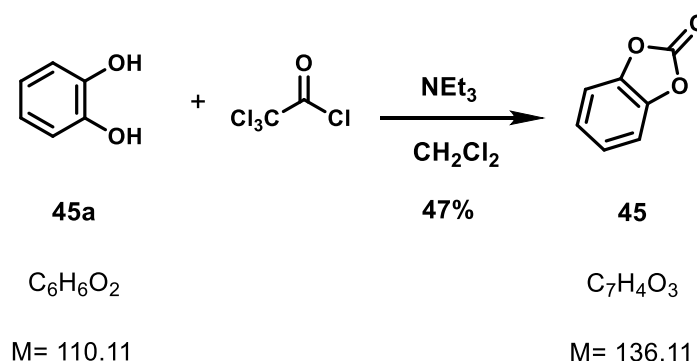
TLC: 0.5 (EtOAc: hexane, 2:3)

¹H NMR (200 MHz, CDCl₃): δ 1.87 (ddt, J = 14.7, 10.8, 4.1 Hz, 1H, H3), 2.18 (dtd, J = 14.1, 5.0, 4.1 Hz, 1H, H3), 2.59 (dt, J = 16.2, 4.8 Hz, 1H, H4), 2.85 (ddd, J = 15.3, 10.8, 4.0 Hz, 1H, H4), 5.10 (dt, J = 8.2, 4.3 Hz, 1H, H2), 5.60 (d, J = 7.9 Hz, 1H, H1), 7.08 – 7.16 (m, 1H, Ar-CH), 7.18 – 7.34 (m, 3H, Ar-CH).

¹³C NMR (50 MHz, CDCl₃): δ 23.7 (t, C4), 27.1 (t, C3), 75.5 (d, C1), 75.7 (d, C2, 127.3 (d, Ar-CH), 128.7 (d, Ar-CH), 129.4 (s, C4a), 129.8 (d, Ar-CH), 130.8 (d, Ar-CH), 138.2 (s, C8a), 154.8 (s, CO).

5.10 Elaboration of carbonate formation of diols

5.10.1 1,3-Benzodioxol-2-one (45)



The reaction was performed according to GP8. Pyrocatechol (45a) (220 mg, 2 mmol) dissolved in 10 mL of dry DCM was reacted with trichloroacetyl chloride (799 mg, 4.4 mmol, 2.2 eq) in presence of triethylamine (667 mg, 6.6 mmol, 3.3 eq). Heating of the reaction mixture was necessary in order to complete the reaction. The carbonate ester formed was not stable for a long time and decomposition was observed on storage at room temperature. Cyclic carbonate **45**^{46,222} was purified by MPLC using EtOAc and hexane mixture (10:1) as eluent.

Yield: 48% (130mg, 0.177 mmol)

²²² [222] A. Ghinet, B. Rigo, J.-P. Hénichart, D. Le Broc-Ryckewaert, J. Pommery, N. Pommery, X. Thuru, B. Quesnel and P. Gautret, *Bioorganic & Medicinal Chemistry* **2011**, *19*, 6042-6054.

Appearance: brown solid

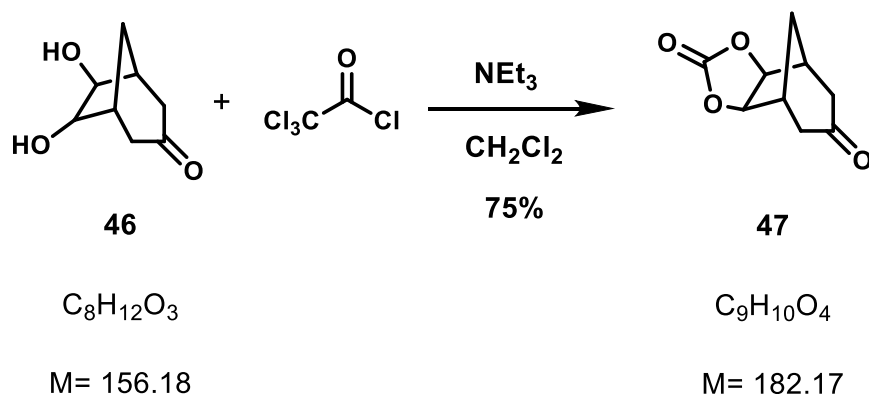
m.p.: 120-122 °C (Lit. 120-122 °C and 119-120 °C²²³)

TLC: 0.4 (EtOAc: hexane, 2: 8)

¹H NMR (200 MHz, CDCl₃): δ 7.21-7.29 (m, 4H).

¹³C NMR (50 MHz, CDCl₃): δ 110.4 (d, Ar-CH), 124.9 (d, Ar-CH), 143.2 (s, CH-C-O), 151.2 (s, CO).

5.10.2 (3aR,4R,8S,8aS)-Tetrahydro-3aH-4,8-methanocyclohepta[d][1,3]dioxole-2,6(7H)-dione (**47**)



Carba diol **46** (16 mg, 0.102 mmol) dissolved in 3 mL of dry DCM was reacted with trichloroacetyl chloride (41 mg, 0.23 mmol, 2.2 eq) in presence of triethylamine (34mg, 0.34 mmol, 3.3 eq). The reaction was performed according to GP11 and product was purified by MPLC using EtOAc and hexane gradient mixture as eluent. Cyclic carbonate **47** was isolated as colorless solid (14 mg, 0.08 mmol).

Yield: 75% (14 mg, 0.08 mmol)

Appearance: colorless solid

m.p.: 164-166 °C

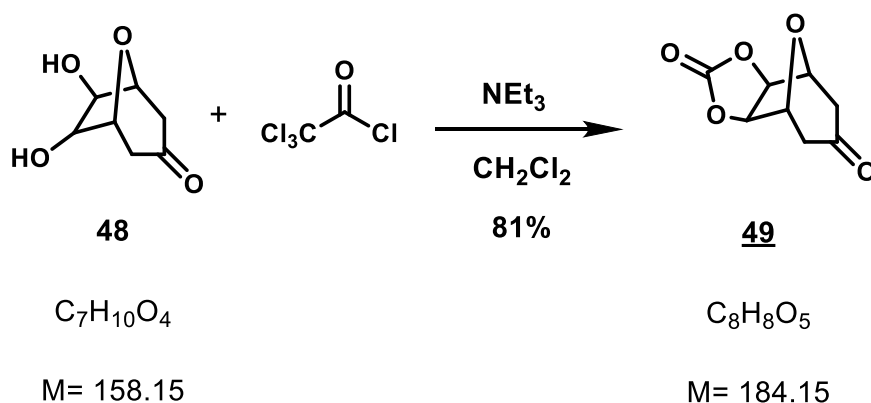
TLC: 0.4 (EtOAc: hexane, 4: 6)

²²³ [223] K. R. Fountain and M. Pierschbacher, *Journal of Organic Chemistry* **1976**, *41*, 2039-2042.

¹H NMR (200 MHz, CDCl₃): δ 1.96 (t, J = 13.2 Hz, 1H), 2.14 – 2.32 (m, 1H), 2.36 – 2.67 (m, 4H), 2.80 (d, J = 4.0 Hz, 2H), 4.85 (d, J = 1.4 Hz, 2H).

¹³C NMR (50 MHz, CDCl₃): δ 30.8 (t, CH₂), 40.7 (t, CH₂), 45.9 (d, CH), 83.8 (d, CH), 154.5 (s, OCOO), 206.8 (s, CCOC).

5.10.3 (3aR,4R,8S,8aS)-Tetrahydro-3aH-4,8-epoxycyclohepta[d][1,3]dioxole-2,6(7H)-dione (49)



Oxo diol **48** (30 mg, 0.19 mmol) was dissolved in 3 mL of dry DCM. Triethylamine (63.45 mg, 0.627 mmol, 3.3 eq) was added in ice bath at -10°C under N₂ atmosphere. Then, trichloroacetyl chloride (76 mg, 0.42 mmol, 2.2 eq) in 1 mL of DCM was added dropwise via a syringe. The reaction was performed according to GP11. Product was purified by MPLC using EtOAc and hexane gradient mixture as eluent. Cyclic carbonate **49** was isolated as colorless solid (28 mg, 0.15 mmol).

Yield: 81% (28 mg, 0.15 mmol)

Appearance: colorless solid

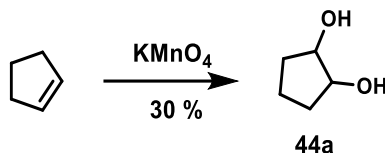
m.p.: 173-175 °C

TLC: 0.4 (EtOAc: hexane, 3: 7)

¹H NMR (200 MHz, CDCl₃): δ 2.42 (s, 1H), 2.50 (d, J = 0.9 Hz, 1H), 2.81 (d, J = 6.1 Hz, 1H), 2.89 (d, J = 6.1 Hz, 1H), 4.81 (d, J = 6.2 Hz, 2H), 4.98 (s, 2H).

¹³C NMR (50 MHz, CDCl₃): δ 45.1 (d, CH), 79.6 (d, CH), 82.3 (t, CH₂), 153.6 (s, OCOO), 202.3 (s, CCOC).

5.10.4 Cyclopentane-1,2-diol (50a)



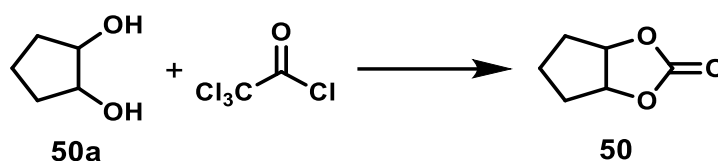
Cyclopentene diol **44a** was prepared according to the literature²²⁴ from cyclopentene (13.62 g, 0.2 mol).

Yield: 31% (6.3 g, 0.062 mol)

Appearance: colorless glassy solid, highly hygroscopic

m.p.: 29-31 °C, Lit 32.18°C

5.10.5 Tetrahydro-4H-cyclopenta[d][1,3]dioxol-2-one (50)



Cyclopentane diol (**50a**) (200 mg, 1.96 mmol) dissolved in 3 mL of dry DCM was reacted with trichloroacetyl chloride (784.36 mg, 4.313 mmol, 2.2 eq) in presence of triethylamine (1091 mg, 10.8 mmol, 5.5 eq). The reaction was carried out using standard carbonate formation procedure and the product was purified by MPLC using EtOAc and hexane gradient mixture as eluent. Cyclic carbonate **50**²²⁵ was isolated as colorless solid (197 mg, 1.54 mmol).

Yield: 34% (85 mg, 0.66 mol)

Appearance: colorless solid

²²⁴ [224] J. E. Taylor, T. E. Janini and O. C. Elmer, *Organic Process Research & Development* **1998**, 2, 147-150.

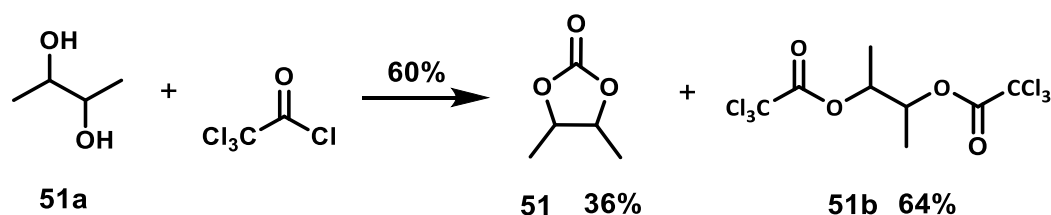
²²⁵ [225] X. Yang, J. Wu, X. Mao, T. F. Jamison and T. A. Hatton, *Chemical Communications* **2014**, 50, 3245-3248.

m.p.: 34-36 °C, Lit 33-35°C²²⁶

¹H NMR (200 MHz, CDCl₃): δ 1.65-1.75 (m, 2H, CH₂ 3/4/5, CP-ring), 1.81- 1.89 (m, 2H, CH₂ 3/4/5, CP-ring), 2.10-2.19 (m, 2H, CH₂ 3/4/5, CP-ring), 5.11 (m, 2H, H1+H2, OCHCHO).

¹³C NMR (50 MHz, CDCl₃): δ 21.6 (t, C4), 33.8 (t, C3+C5), 82.3 (d, C1+C2), 155.9 (s, CO).

5.10.6 4,5-Dimethyl-1,3-dioxolan-2-one (51)



Butane-2,3-diol (**51a**) (180 mg, 2 mmol) dissolved in 3 mL of dry DCM was reacted with trichloroacetyl chloride (800 mg, 4.4 mmol, 2.2 eq) in presence of triethylamine (668 mg, 6.6 mmol, 3.3 eq). The reaction was performed according to GP11 and product was purified by MPLC using EtOAc and hexane gradient mixture as eluent. Cyclic carbonate **51** was isolated as colorless liquid (30 mg, 0.256 mmol) in 13% yield. The major product of reaction was diester of trichloroacetyl chloride **51b**, which was formed in 64% and monoester **51b** was formed in 36% yield respectively.

Yield: 36% (50 mg, 0.43 mmol)

4,5-Dimethyl-1,3-dioxolan-2-one (51)²²⁶

Appearance: yellowish viscous liquid

¹H NMR (200 MHz, CDCl₃): δ 1.35 – 1.46 (m, 6H), 4.15 – 4.39 (m, 2H).

¹³C NMR (50 MHz, CDCl₃): δ 18.4 (q, CH₃), 79.9 (d, CH), 154.4 (s, CO).

Butane-2,3-diyl bis(2,2,2-trichloroacetate) (51b)

Appearance: colorless liquid

¹H NMR (200 MHz, CDCl₃): δ 1.34 – 1.52 (m, 6H), 5.13 – 5.35 (m, 2H).

²²⁶ [226] B. Gabriele, R. Mancuso, G. Salerno, L. Veltri, M. Costa and A. Dibenedetto, *ChemSusChem* **2011**, *4*, 1778-1786.

Yield: 52% (294 mg, 2.07 mmol)

Appearance: colorless solid

m.p.: 34-36 °C (Lit 34-35 °C),²²⁶ 34-37 °C²³⁰

¹H NMR (200 MHz, CDCl₃): δ 1.19 – 1.38 (m, 2H), 1.39 – 1.59 (m, 2H), 1.63 – 1.92 (m, 4H), 4.61 (t, J = 4.0 Hz, 2H).

¹³C NMR (50 MHz, CDCl₃): δ 19.0 (t, CH₂), 26.6 (t, CH₂), 75.8 (d, CH), 155.4 (s, CO).

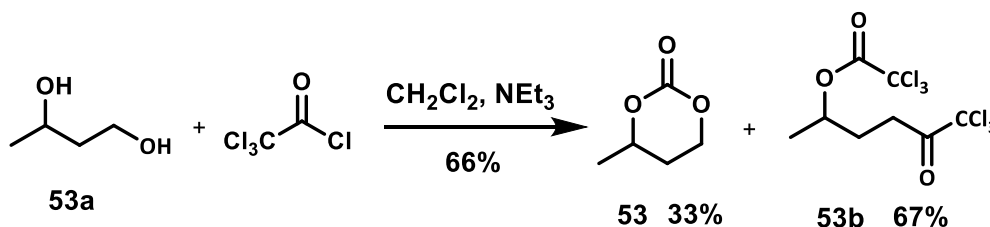
Cyclohexane-1,2-diyl bis(2,2,2-trichloroacetate) **52b**

Appearance: colorless solid

¹H NMR (200 MHz, CDCl₃): δ 1.41 – 1.58 (m, 2H), 1.59 – 1.91 (m, 4H), 1.91 – 2.20 (m, 1H) 5.08-5.30 (t, J = 4.0 Hz, 2H).

¹³C NMR (50 MHz, CDCl₃): δ 29.3 (t, CH₂), 78.2 (d, CH), 89.7 (s, CCl₃), 161.1 (s, CO).

5.10.9 4-methyl-1,3-dioxan-2-one (**53**)



Butane-1,3-diol (**53a**) (180 mg, 2 mmol) dissolved in 3 mL of dry DCM was reacted with trichloroacetyl chloride (800 mg, 4.4 mmol, 2.2 eq) in presence of triethylamine (668 mg, 6.6 mmol, 3.3 eq). The reaction was performed according to GP11 and product was purified by MPLC using EtOAc and hexane gradient mixture as eluent. Cyclic carbonate **53** was isolated as colorless liquid (46 mg, 0.4 mmol) in 33% yield. Major product of reaction was diester of trichloroacetyl chloride **53b** and was formed in 67% yield.

Yield: 33% (46 mg, 0.4 mmol)

Appearance: yellowish oil

4-Methyl-1,3-dioxan-2-one1 (53)^{231, 232, 226}

¹H NMR (200 MHz, CDCl₃): δ 1.44 (s, 3H), 1.93 – 2.15 (m, 2H), 4.37 – 4.46 (m, 2H), 4.61 – 4.69 (m, 1H).

¹³C NMR (50 MHz, CDCl₃): δ 21.1 (q, CH₃), 28.6 (t, CH₂), 67.2 (t, CH₂), 76.0 (d, CH), 149.4 (s, CO).

Butane-1,3-diyl bis(2,2,2-trichloroacetate) (53b)

Appearance: colorless liquid

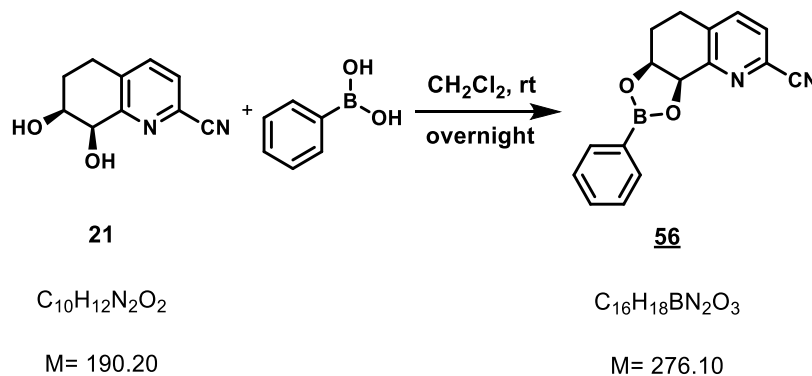
¹H NMR (200 MHz, CDCl₃): δ 1.41 (d, J = 6.3 Hz, 3H), 1.73 – 1.98 (m, 1H), 1.98 – 2.18 (m, 1H), 4.24 – 4.50 (m, 2H), 4.50 – 4.70 (m, 1H).

¹³C NMR (50 MHz, CDCl₃): δ 19.4 (q, CH₃), 34.0 (t, CH₂), 64.9 (t, CH₂), 73.5 (d, CH), 89.5 (s, CCl₃), 89.9 (s, CCl₃), 161.2 (s, CO), 161.8 (s, CO).

²³¹ [231] M. Fujiwara, A. Baba and H. Matsuda, *Journal of Heterocyclic Chemistry* **1989**, 26, 1659-1663.
²³² [232] K. Pihlaja and K. Rossi, *Acta Chem. Scand., Ser. B* **1983**, B37, 289-296.

5.11 Boronic ester synthesis of diols

5.11.1 (3a*S*,9b*R*)-2-phenyl-3a,4,5,9b-tetrahydro-[1,3,2]dioxaborolo[4,5-*h*]quinoline-8-carbonitrile (**56**)



A 50 mL round-bottomed flask was charged with reduced naphthalene diol **21** (100 mg, 0.53 mmol) and reacted with phenylboronic acid (1.2 eq, 78 mg, 0.64 mmol) in 3 mL DCM at room temperature under N_2 atmosphere for 6 hours. After completion of the reaction, phenylboronic ester was purified by MPLC using EtOAc and hexane as eluent (1:1).

Yield: 90% (132 mg, 0.48 mmol)

Appearance: Beige solid

m.p.: 197-199 °C

TLC: 0.0 (EtOAc: hexane, 0: 0)

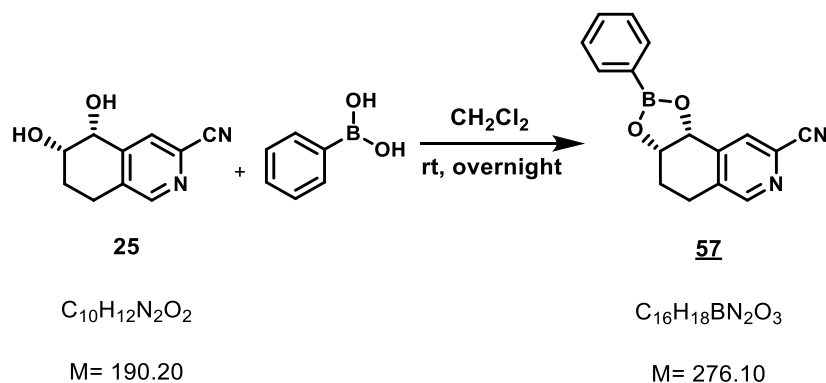
$\alpha_{\text{D}}^{20} =$ +152 (c=1.01, EtOAc)

^1H NMR (200 MHz, CDCl_3): δ 1.96 (ddt, $J = 14.3, 11.7, 3.9$ Hz, 1H), 2.35 (dq, $J = 14.4, 4.1$ Hz, 1H), 2.71 (dt, $J = 16.2, 4.3$ Hz, 1H), 3.01 (ddd, $J = 15.8, 11.7, 3.8$ Hz, 1H), 5.19 (dt, $J = 7.8, 3.8$ Hz, 1H), 5.62 (d, $J = 7.9$ Hz, 1H), 7.36 (dd, $J = 7.9, 6.4$ Hz, 2H), 7.42 – 7.53 (m, 1H), 7.60 (d, $J = 1.8$ Hz, 2H), 7.77 – 7.88 (m, 2H).

^{13}C NMR (101 MHz, CDCl_3): δ 23.8 (t, C6), 28.9 (t, C5), 75.7 (d, C7), 77.1 (d, C8), 117.2 (s, CN), 128.0 (d, Ar-CH), 128.0 (d, Ar-C-B), 131.9 (d, C3),

132.5 (s, C2), 135.2 (d, Ar-CH), 137.4 (d, C4), 138.7 (s, C4a), 156.0 (s, C8a).

5.11.2 (3aS,9bR)-2-phenyl-3a,4,5,9b-tetrahydro-[1,3,2]dioxaborolo[4,5-f]isoquinoline-8-carbonitrile (**57**)



A 50 mL round-bottomed flask was charged with reduced naphthalene diol **25** (50 mg, 0.26 mmol) and reacted with phenylboronic acid (1.2 eq, 78 mg, 0.64 mmol) in DCM at room temperature under N_2 atmosphere. After completion of the reaction, phenylboronic ester was purified by MPLC using EtOAc and hexane gradient mixture as eluent.

Yield: 77 % (55 mg, 0.20 mmol)

Appearance: beige solid

m.p.:

TLC: 0.0 (EtOAc: hexane, 0: 0)

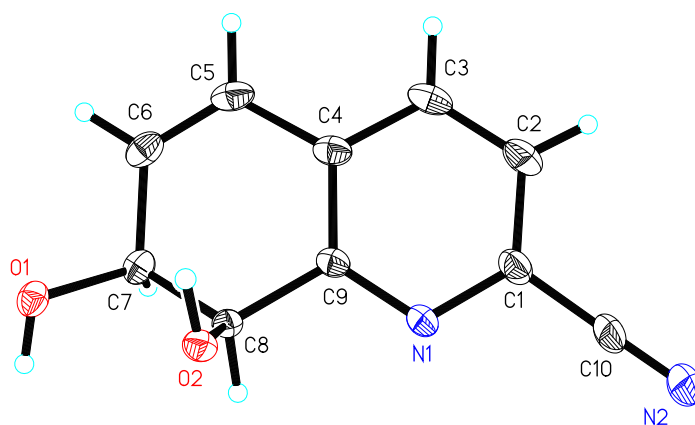
^1H NMR (200 MHz, CDCl_3): δ 2.00 – 2.27 (m, 2H, H7), 2.67 (ddd, $J = 16.4, 6.7, 4.4$ Hz, 1H, H8), 2.93 (ddd, $J = 16.5, 8.3, 4.9$ Hz, 1H, H8), 5.08 (ddd, $J = 7.9, 5.9, 4.7$ Hz, 1H, H6), 5.44 (d, $J = 7.9$ Hz, 1H, H5), 7.32 – 7.64 (m, 3H, Ar-H), 7.75 – 7.85 (m, 2H, Ar-H), 7.87 (s, 1H, H4), 8.52 (s, 1H, H1).

^{13}C NMR (50 MHz, CDCl_3): δ 21.8 (t, c7), 28.1 (t, C8), 73.6 (d, C5), 75.1 (d, C6), 117.2 (s, CN), 127.9 (d, Ar-C), 128.0 (d, Ar-C), 129.1 (d, C4), 132.0 (Ar-C), 132.19 (Ar-C), 132.7 (d, C3), 134.95 (d, Ar-C),

135.62 (d, Ar-C), 137.5 (s, C8a), 144.4 (d, C4a), 150.9 (s, C1).

6 Crystallographic Data

6.1 Crystal structure report of *cis*-(7*S*,8*R*)-7,8-dihydroxy-7,8-dihydroquinoline-2-carbonitrile (**10**)



Crystal structure 1154 mm ZF036F3 - C₁₀H₈N₂O₂

Single-crystal X-ray diffraction data for compound **10** were collected with a Bruker Smart APEX CCD 3-circle diffractometer operating with graphite monochromatized Mo-K α radiation. Structure solution and refinement was performed with Bruker AXS SHELXTL software.

6.1.1 Crystal Data and Structure Refinement for compound **10**

Identification code	1154m
Empirical formula	C ₁₀ H ₈ N ₂ O ₂
Formula weight	188.18
Temperature	297(2) K
Wavelength	0.71073 Å
Crystal system, space group	Orthorhombic, P2(1)2(1)2(1)
Unit cell dimensions	a = 6.7221(6) Å alpha = 90
deg.	b = 8.2832(7) Å beta = 90
deg.	c = 32.073(3) Å gamma = 90

deg.	
Volume	1785.8(3) Å ³
Z, Calculated density	8, 1.400 Mg/m ³
Absorption coefficient	0.100 mm ⁻¹
F(000)	784
Crystal size	0.45 x 0.30 x 0.26 mm
Diffractometer	Bruker Smart APEX CCD 3-
circle (sealed X-ray	tube, Mo Kalfa rad.,
graphite monochromator	detector.distance 50 mm,
512x512 pixels)	
Scan type / width / speed	ome-scan frames /
dome=0.3deg / 20sec per frame	
	full sphere data collection,
4 x 606 frames	
Theta range for data collection	2.54 to 29.99 deg.
Index ranges	-8<=h<=9, -11<=k<=11, -
25<=l<=45	
Reflections collected / unique	13843 / 3003 [R(int) =
0.0226]	
Completeness to theta = 29.99	99.7%
Absorption correction	Multi-scan (program SADABS;
Sheldrick, 1996)	
Max. and min. transmission	1.00 and 0.82
Structure solution	Direct methods (program
SHELXS97)	
Refinement method	Full-matrix least-squares on
F2 (prg SHELXL97)	
Data / restraints / parameters	3003 / 216 / 269
Goodness-of-fit on F2	1.055
Final R indices [I>2sigma(I)]	R1 = 0.0439, wR2 = 0.1119
R indices (all data)	R1 = 0.0496, wR2 = 0.1169
Absolute structure parameter	-1.3(11)
Largest diff. peak and hole	0.34 and -0.18 eÅ ⁻³

$$R1 = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}, \quad wR2 = \left[\frac{\sum (w(F_o^2 - F_c^2))^2}{\sum (w(F_o^2))^2} \right]^{\frac{1}{2}}$$

6.1.2 Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 10

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
O (1)	6426 (2)	4089 (2)	2209 (1)	44 (1)
O (2)	3328 (2)	3093 (2)	1719 (1)	38 (1)
N (1)	2822 (2)	5168 (2)	927 (1)	43 (1)
N (2)	-271 (3)	6333 (4)	147 (1)	84 (1)
C (1)	2843 (3)	5137 (3)	505 (1)	50 (1)
C (2)	4399 (3)	4538 (3)	270 (1)	60 (1)
C (3)	6049 (3)	3970 (3)	477 (1)	59 (1)
C (4)	6121 (3)	4031 (2)	912 (1)	46 (1)
C (5)	7889 (3)	3601 (2)	1149 (1)	53 (1)
C (6)	8021 (3)	3920 (2)	1553 (1)	50 (1)
C (7)	6394 (2)	4724 (2)	1796 (1)	41 (1)
C (8)	4329 (2)	4535 (2)	1595 (1)	36 (1)
C (9)	4425 (2)	4611 (2)	1122 (1)	38 (1)
C (10)	1097 (3)	5811 (3)	307 (1)	63 (1)
O (1')	7755 (2)	-2295 (2)	2441 (1)	47 (1)
O (2')	6243 (2)	837 (1)	2317 (1)	40 (1)
N (1')	5392 (2)	245 (2)	1483 (1)	35 (1)
N (2')	1652 (3)	902 (3)	767 (1)	72 (1)
C (1')	4861 (3)	-253 (2)	1097 (1)	40 (1)
C (2')	5967 (3)	-1316 (2)	859 (1)	49 (1)
C (3')	7730 (3)	-1882 (2)	1021 (1)	48 (1)
C (4')	8329 (2)	-1395 (2)	1418 (1)	38 (1)
C (5')	10165 (3)	-1963 (2)	1608 (1)	46 (1)
C (6')	10511 (3)	-1769 (2)	2012 (1)	49 (1)
C (7')	8993 (3)	-1024 (2)	2295 (1)	39 (1)
C (8')	7797 (2)	264 (2)	2060 (1)	34 (1)
C (9')	7080 (2)	-334 (2)	1639 (1)	32 (1)
C (10')	3042 (3)	409 (3)	925 (1)	50 (1)

6.1.3 Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 10

Hydrogen atoms inserted in idealized positions and refined riding with the atoms to which they were bonded. All H atoms had $U_{\text{iso}} = U_{\text{eq}} \times 1.2$ of their carrier atoms.

	x	y	z	U_{eq}
H(1O)	5710 (40)	4680 (30)	2364 (7)	61 (7)
H(2O)	4090 (30)	2320 (20)	1650 (7)	63 (7)
H(2)	4333	4520	-20	72
H(3)	7114	3546	328	71
H(5)	8944	3095	1015	64
H(6)	9179	3630	1693	60
H(7)	6697	5880	1812	49
H(8)	3515	5448	1688	43
H(1'O)	7410 (40)	-2050 (30)	2687 (5)	62 (7)
H(2'O)	6170 (30)	1847 (17)	2280 (6)	40 (5)
H(2')	5532	-1641	597	59
H(3')	8518	-2586	867	58
H(5')	11115	-2471	1443	56
H(6')	11723	-2103	2122	59
H(7')	9670	-521	2533	47
H(8')	8694	1175	2008	41

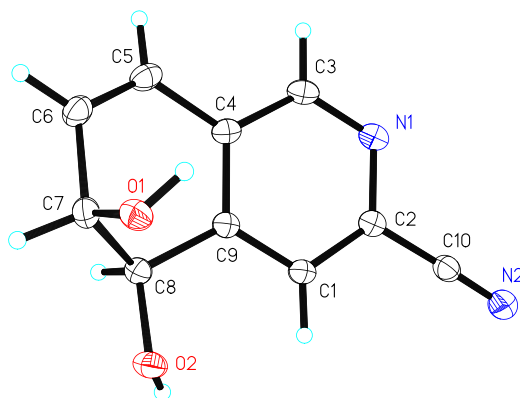
6.1.5 Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 10

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O(1)	49(1)	36(1)	47(1)	0(1)	-9(1)	5(1)
O(2)	36(1)	42(1)	38(1)	1(1)	5(1)	1(1)
N(1)	45(1)	54(1)	32(1)	1(1)	5(1)	1(1)
N(2)	72(1)	45(2)	37(1)	10(1)	0(1)	10(2)
C(1)	53(1)	65(1)	33(1)	1(1)	7(1)	-5(1)
C(2)	69(1)	74(1)	38(1)	-3(1)	18(1)	-4(1)
C(3)	61(1)	65(1)	52(1)	-8(1)	27(1)	-1(1)
C(4)	45(1)	44(1)	49(1)	1(1)	15(1)	1(1)
C(5)	41(1)	48(1)	71(1)	5(1)	19(1)	7(1)
C(6)	34(1)	45(1)	70(1)	14(1)	1(1)	-1(1)
C(7)	42(1)	32(1)	50(1)	4(1)	-5(1)	-2(1)
C(8)	37(1)	35(1)	35(1)	-1(1)	2(1)	6(1)
C(9)	40(1)	38(1)	37(1)	0(1)	8(1)	-1(1)
C(10)	64(1)	97(2)	28(1)	4(1)	4(1)	-1(1)
O(1')	69(1)	36(1)	37(1)	3(1)	5(1)	2(1)
O(2')	53(1)	31(1)	36(1)	-3(1)	1(1)	4(1)
N(1')	38(1)	35(1)	33(1)	0(1)	-2(1)	-1(1)
N(2')	63(1)	90(2)	64(1)	-6(1)	-23(1)	9(1)
C(1')	43(1)	43(1)	34(1)	3(1)	-7(1)	-5(1)
C(2')	65(1)	54(1)	29(1)	-3(1)	-3(1)	-2(1)
C(3')	62(1)	50(1)	33(1)	-4(1)	8(1)	9(1)
C(4')	43(1)	37(1)	33(1)	4(1)	6(1)	3(1)
C(5')	43(1)	49(1)	47(1)	8(1)	8(1)	13(1)
C(6')	43(1)	52(1)	52(1)	7(1)	-5(1)	11(1)
C(7')	47(1)	36(1)	35(1)	1(1)	-9(1)	2(1)
C(8')	40(1)	29(1)	33(1)	0(1)	-5(1)	-1(1)
C(9')	36(1)	30(1)	29(1)	3(1)	0(1)	-2(1)
C(10')	51(1)	58(1)	42(1)	-2(1)	-11(1)	-4(1)

6.2 *cis*-(5*R*,6*S*)-5,6-dihydroxy-5,6-dihydroisoquinoline-3-carbonitrile (**15**)

1215ttin, C₁₀H₈N₂O₂

Single-crystal X-ray diffraction data for compound **15** were collected with a Bruker Smart APEX CCD 3-circle diffractometer operating with graphite monochromatized Mo-K α radiation. Structure solution and refinement was performed with Bruker AXS SHELXTL software.

6.2.1 Crystal Data and Structure Refinement for compound **15**

Identification code	1215ttin
Empirical formula	C ₁₀ H ₈ N ₂ O ₂
Formula weight	188.18
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 8.4435(5) Å alpha = 90 deg.
	b = 6.4126(4) Å beta =
110.2750(10) deg.	c = 8.5056(5) Å gamma = 90 deg.
Volume	432.00(5) Å ³

Z, Calculated density	2, 1.447 Mg/m ³
Absorption coefficient	0.104 mm ⁻¹
F(000)	196
Crystal size	0.60 x 0.40 x 0.30 mm
Diffractometer	Bruker Smart APEX CCD 3-
circle (sealed X-ray	tube, Mo Kalfa rad.,
graphite monochromator	detector.distance 50 mm,
512x512 pixels)	
Scan type / width / speed	ome-scan frames /
dome=0.3deg / 10sec per fram	full sphere data collection,
3 x 600 frames	
Theta range for data collection	2.55 to 29.98 deg.
Index ranges	-11<=h<=11, -8<=k<=8, -
11<=l<=11	
Reflections collected / unique	4698 / 2427 [R(int) =
0.0146]	
Completeness to theta = 29.98	99.0%
Absorption correction	Multi-scan (program SADABS;
Sheldrick, 1996)	
Max. and min. transmission	0.97 and 0.87
Structure solution	Direct methods (program
SHELXS97)	
Refinement method	Full-matrix least-squares on
F2 (prg SHELXL97)	
Data / restraints / parameters	2427 / 37 / 135
Goodness-of-fit on F2	1.080
Final R indices [I>2sigma(I)]	R1 = 0.0355, wR2 = 0.0937
R indices (all data)	R1 = 0.0358, wR2 = 0.0943
Absolute structure parameter	0.5(7)
Largest diff. peak and hole	0.46 and -0.16 eA ⁻³

$$R1 = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}, \quad wR2 = \left[\frac{\sum (w(F_o^2 - F_c^2))^2}{\sum (w(F_o^2))^2} \right]^{1/2}$$

6.2.3 Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for 15

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
O (1)	7362 (1)	995 (1)	9387 (1)	18 (1)
O (2)	5329 (1)	3847 (1)	10312 (1)	21 (1)
N (1)	3713 (1)	5001 (1)	4040 (1)	18 (1)
N (2)	-422 (1)	5105 (2)	3790 (1)	28 (1)
C (1)	3596 (1)	4769 (2)	6848 (1)	17 (1)
C (2)	2852 (1)	4918 (2)	5106 (1)	17 (1)
C (3)	5397 (1)	4950 (2)	4716 (1)	18 (1)
C (4)	6275 (1)	4819 (2)	6445 (1)	16 (1)
C (5)	8116 (1)	4642 (2)	7138 (1)	20 (1)
C (6)	8876 (1)	3852 (2)	8678 (1)	21 (1)
C (7)	7844 (1)	3144 (2)	9721 (1)	19 (1)
C (8)	6315 (1)	4572 (2)	9387 (1)	17 (1)
C (9)	5347 (1)	4714 (2)	7528 (1)	15 (1)
C (10)	1028 (1)	5015 (2)	4352 (1)	20 (1)
H (10)	7100 (20)	800 (30)	8350 (20)	31 (4)
H (20)	4710 (20)	4700 (40)	10330 (20)	34 (4)
H (1)	2927	4708	7543	20
H (3)	6031	5006	3987	21
H (5)	8776	5088	6495	24
H (6)	10071	3737	9116	25
H (7)	8560	3274	10930	22
H (8)	6733	5999	9801	21

Most hydrogen atoms inserted in idealized positions

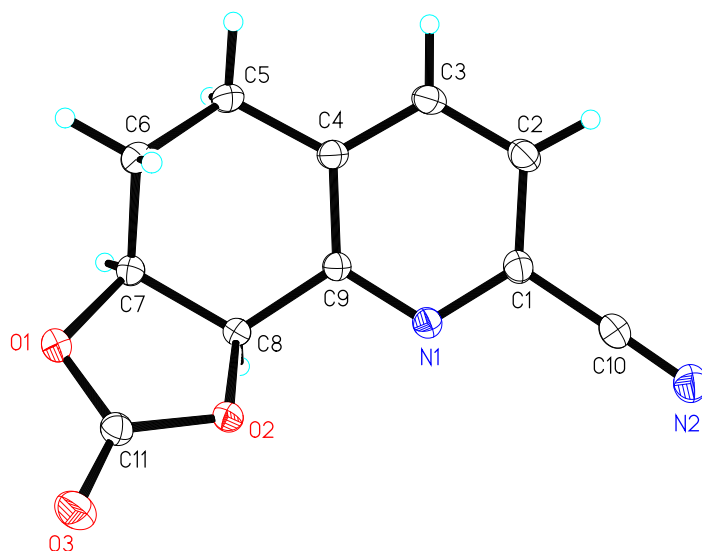
6.2.5 Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 15

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O (1)	21 (1)	19 (1)	16 (1)	1 (1)	7 (1)	2 (1)
O (2)	23 (1)	25 (1)	19 (1)	2 (1)	11 (1)	4 (1)
N (1)	23 (1)	14 (1)	18 (1)	2 (1)	9 (1)	2 (1)
N (2)	24 (1)	38 (1)	22 (1)	-2 (1)	8 (1)	-2 (1)
C (1)	18 (1)	17 (1)	16 (1)	0 (1)	8 (1)	0 (1)
C (2)	20 (1)	15 (1)	17 (1)	1 (1)	7 (1)	1 (1)
C (3)	24 (1)	14 (1)	19 (1)	3 (1)	12 (1)	2 (1)
C (4)	18 (1)	13 (1)	20 (1)	1 (1)	9 (1)	0 (1)
C (5)	19 (1)	18 (1)	27 (1)	-1 (1)	12 (1)	-3 (1)
C (6)	16 (1)	22 (1)	25 (1)	-4 (1)	8 (1)	-4 (1)
C (7)	17 (1)	21 (1)	17 (1)	-3 (1)	4 (1)	-1 (1)
C (8)	18 (1)	19 (1)	16 (1)	-2 (1)	7 (1)	-1 (1)
C (9)	18 (1)	13 (1)	17 (1)	-1 (1)	7 (1)	-1 (1)
C (10)	23 (1)	22 (1)	16 (1)	-1 (1)	7 (1)	-1 (1)

6.3 (3a*S*,9b*R*)-2-oxo-3a,9b-tetrahydro- [1,3]dioxolo[4,5-*h*]quinoline-8-carbonitrile (**39**)

1214mm TT absolute structure C₁₁H₈N₂O₃

Single-crystal X-ray diffraction data for compound **39** were collected with a Bruker Smart APEX CCD 3-circle diffractometer operating with graphite monochromatized Mo-K α radiation. Structure solution and refinement was performed with Bruker AXS SHELXTL software.

6.3.1 Crystal Data and Structure Refinement for compound **39**

Identification code	1214ttin
Empirical formula	C ₁₁ H ₈ N ₂ O ₃
Formula weight	216.19
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 6.7117(5) Å alpha = 90
deg.	b = 7.1543(6) Å beta =
101.880(2) deg.	c = 9.8008(8) Å gamma = 90
deg.	

Volume	460.53(6) A ³
Z, Calculated density	2, 1.559 Mg/m ³
Absorption coefficient	0.116 mm ⁻¹
F(000)	224
Crystal size	0.58 x 0.34 x 0.20 mm
Diffractometer	Bruker Smart APEX CCD 3-
circle (sealed X-ray	tube, Mo Kalfa rad.,
graphite monochromator	detector.distance 50 mm,
512x512 pixels)	
Scan type / width / speed	ome-scan frames /
dome=0.3deg / 10sec per fram	hemisphere data collection,
3 x 600 frames	
Theta range for data collection	3.10 to 29.98 deg.
Index ranges	-9<=h<=9, -10<=k<=10, -
13<=l<=13	
Reflections collected / unique	5036 / 2547 [R(int) =
0.0120]	
Completeness to theta = 29.98	97.9%
Absorption correction	Multi-scan (program SADABS;
Sheldrick, 1996)	
Max. and min. transmission	0.98 and 0.91
Structure solution	Direct methods (program
SHELXS97)	
Refinement method	Full-matrix least-squares on
F2 (prg SHELXL97)	
Data / restraints / parameters	2547 / 1 / 145
Goodness-of-fit on F2	1.066
Final R indices [I>2sigma(I)]	R1 = 0.0298, wR2 = 0.0779
R indices (all data)	R1 = 0.0300, wR2 = 0.0782
Absolute structure parameter	0.4(6)
Largest diff. peak and hole	0.342 and -0.215 eA ⁻³

$$R1 = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}, \quad wR2 = \left[\frac{\sum (w(F_o^2 - F_c^2))^2}{\sum (w(F_o^2))^2} \right]^{1/2}$$

Absolute structure known from the p-iodobenzoate of the diol.

6.3.2 Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 39

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
O(1)	7881 (1)	3796 (1)	9953 (1)	18 (1)
O(2)	5379 (1)	3606 (1)	8072 (1)	14 (1)
O(3)	5292 (1)	1796 (1)	9923 (1)	22 (1)
N(1)	5182 (1)	5011 (1)	5352 (1)	14 (1)
N(2)	2018 (1)	4692 (1)	2195 (1)	20 (1)
C(1)	5400 (2)	5025 (1)	4022 (1)	15 (1)
C(2)	7260 (2)	5194 (1)	3604 (1)	17 (1)
C(3)	8994 (2)	5345 (2)	4647 (1)	18 (1)
C(4)	8841 (1)	5321 (1)	6046 (1)	14 (1)
C(5)	10673 (2)	5431 (2)	7217 (1)	18 (1)
C(6)	10337 (1)	4274 (2)	8463 (1)	16 (1)
C(7)	8506 (1)	5064 (1)	8960 (1)	14 (1)
C(8)	6571 (1)	5210 (1)	7813 (1)	13 (1)
C(9)	6885 (1)	5151 (1)	6334 (1)	13 (1)
C(10)	3520 (2)	4845 (1)	2993 (1)	16 (1)
C(11)	6117 (1)	2976 (2)	9374 (1)	15 (1)
H(2A)	7333	5205	2646	21
H(3A)	10291	5465	4409	21
H(5A)	10929	6750	7505	21
H(5B)	11883	4957	6895	21
H(6A)	10088	2950	8184	19
H(6B)	11560	4334	9223	19
H(7A)	8851	6315	9402	17
H(8A)	5825	6380	7953	15

Hydrogen atoms inserted in idealized positions

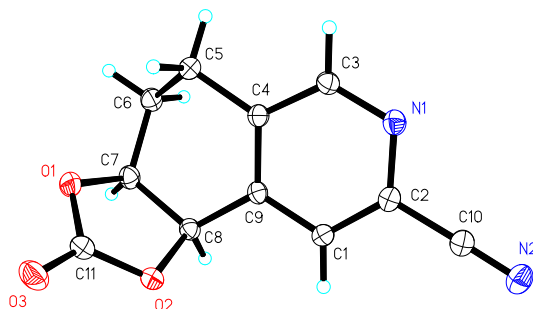
6.3.4 Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 39

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O (1)	15 (1)	25 (1)	13 (1)	4 (1)	3 (1)	-1 (1)
O (2)	14 (1)	17 (1)	12 (1)	1 (1)	3 (1)	-3 (1)
O (3)	21 (1)	26 (1)	21 (1)	6 (1)	8 (1)	0 (1)
N (1)	14 (1)	15 (1)	13 (1)	1 (1)	3 (1)	1 (1)
N (2)	21 (1)	22 (1)	17 (1)	0 (1)	3 (1)	-2 (1)
C (1)	16 (1)	15 (1)	13 (1)	0 (1)	3 (1)	2 (1)
C (2)	18 (1)	21 (1)	14 (1)	2 (1)	6 (1)	2 (1)
C (3)	15 (1)	23 (1)	17 (1)	2 (1)	6 (1)	2 (1)
C (4)	13 (1)	17 (1)	15 (1)	2 (1)	4 (1)	1 (1)
C (5)	12 (1)	25 (1)	17 (1)	3 (1)	3 (1)	-2 (1)
C (6)	12 (1)	20 (1)	15 (1)	2 (1)	2 (1)	0 (1)
C (7)	13 (1)	18 (1)	12 (1)	0 (1)	2 (1)	-2 (1)
C (8)	12 (1)	15 (1)	12 (1)	0 (1)	3 (1)	-1 (1)
C (9)	12 (1)	14 (1)	12 (1)	1 (1)	3 (1)	1 (1)
C (10)	19 (1)	16 (1)	14 (1)	1 (1)	5 (1)	1 (1)
C (11)	14 (1)	19 (1)	13 (1)	1 (1)	4 (1)	3 (1)

6.5 (3*aS*,9*bR*)-2-oxo-3*a*,4,5,9*b*-tetrahydro- [1,3]dioxolo[4,5-*f*]isoquinoline-8-carbonitrile (**40**)

1273mm C₁₁H₈N₂O₃, ZF115F1

6.5.1 Crystal data and structure refinement for compound **40**

Identification code	1273m
Empirical formula	C ₁₁ H ₈ N ₂ O ₃
Formula weight	216.19
Temperature	100 (2) K
Wavelength	0.71073 Å
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 7.9247(3) Å alpha = 90 deg. b = 6.6046(3) Å beta = 103.1710(10) deg. c = 9.5200(4) Å gamma = 90 deg.
Volume	485.16(4) Å ³
Z, Calculated density	2, 1.480 Mg/m ³
Absorption coefficient	0.111 mm ⁻¹
F(000)	224
Crystal size	0.60 x 0.48 x 0.24 mm,
colorless plate	
Diffractometer	Bruker SMART CCD 3-circle
(sealed X-ray	tube, Mo Kalfa rad.,
graphite monochromator	CCD in 512x512 pixel mode)

Scan type / width / speed 6 sec. per frame	w-scan frames / dw = 0.30 /
5 x 600 frames	full sphere data collection,
Theta range for data collection	3.03 to 29.97 deg.
Index ranges 13<=l<=13	-11<=h<=11, -9<=k<=9, -
Reflections collected / unique 0.0154]	8893 / 2794 [R(int) =
Completeness to theta = 29.97	99.3%
Absorption correction Sheldrick, 1996)	Multi-scan (program SADABS;
Max. and min. transmission	0.97 and 0.89
Structure solution SHELXS97)	Direct methods (program
Absorption correction equivalents	Semi-empirical from
Refinement method F2 (prg SHELXL97)	Full-matrix least-squares on
Data / restraints / parameters	2794 / 45 / 145
Goodness-of-fit on F2	1.079
Final R indices [I>2sigma(I)]	R1 = 0.0321, wR2 = 0.0867
R indices (all data)	R1 = 0.0324, wR2 = 0.0872
Absolute structure parameter	-0.8(6)
Largest diff. peak and hole	0.437 and -0.196 eA-3

$$R1 = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}, \quad wR2 = \left[\frac{\sum (w(F_o^2 - F_c^2))^2}{\sum (w(F_o^2))^2} \right]^{1/2}$$

Absolute structure known from the p-iodobenzoate of the diol.

6.5.3 Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 40

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
O (1)	9696 (1)	992 (1)	1385 (1)	22 (1)
O (2)	9590 (1)	585 (1)	3682 (1)	19 (1)
O (3)	10459 (1)	-1990 (1)	2477 (1)	27 (1)
N (1)	3431 (1)	2463 (1)	3405 (1)	18 (1)
N (2)	3759 (1)	2534 (2)	7053 (1)	24 (1)
C (1)	6468 (1)	2511 (1)	4634 (1)	15 (1)
C (2)	4708 (1)	2472 (1)	4611 (1)	16 (1)
C (3)	3944 (1)	2493 (2)	2163 (1)	18 (1)
C (4)	5675 (1)	2521 (1)	2046 (1)	15 (1)
C (5)	6142 (1)	2581 (2)	605 (1)	18 (1)
C (6)	7778 (1)	3837 (2)	696 (1)	19 (1)
C (7)	9268 (1)	2998 (1)	1828 (1)	18 (1)
C (8)	8863 (1)	2598 (1)	3308 (1)	16 (1)
C (9)	6957 (1)	2541 (1)	3320 (1)	14 (1)
C (10)	4160 (1)	2491 (2)	5968 (1)	18 (1)
C (11)	9960 (1)	-283 (2)	2509 (1)	20 (1)
H (1)	7312	2516	5521	19
H (3)	3071	2496	1294	21
H (5A)	5175	3178	-121	22
H (5B)	6336	1186	295	22
H (6A)	8090	3835	-253	22
H (6B)	7554	5255	938	22
H (7)	10298	3910	1940	21
H (8)	9474	3613	4026	19

Hydrogen atoms inserted in idealized positions

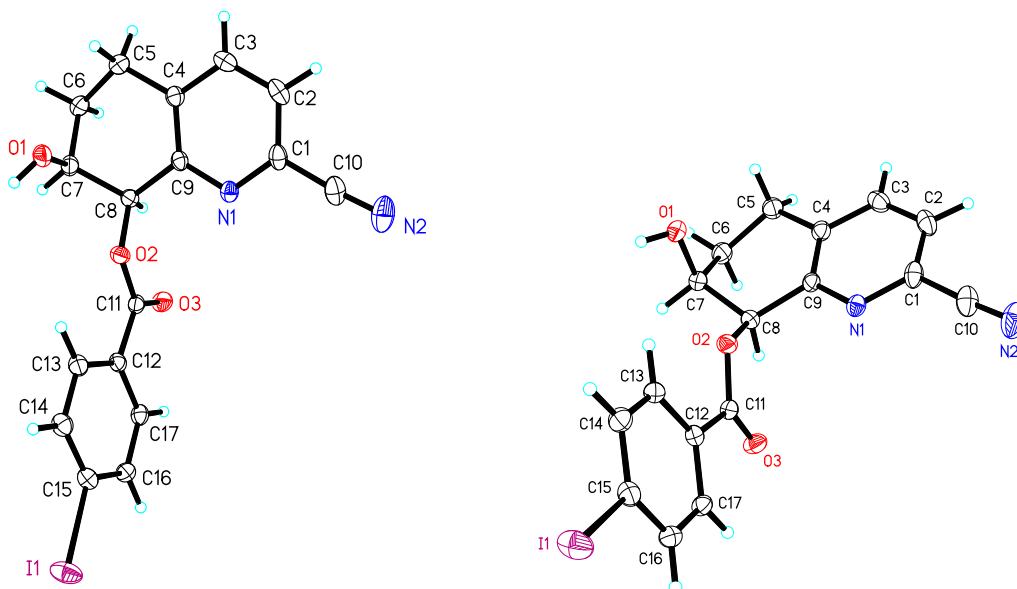
6.5.5 Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound **40**

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O (1)	25 (1)	24 (1)	18 (1)	2 (1)	7 (1)	9 (1)
O (2)	18 (1)	22 (1)	17 (1)	3 (1)	4 (1)	5 (1)
O (3)	26 (1)	23 (1)	30 (1)	-1 (1)	2 (1)	9 (1)
N (1)	17 (1)	19 (1)	19 (1)	0 (1)	6 (1)	-1 (1)
N (2)	30 (1)	21 (1)	23 (1)	1 (1)	10 (1)	0 (1)
C (1)	17 (1)	14 (1)	15 (1)	0 (1)	4 (1)	0 (1)
C (2)	19 (1)	13 (1)	17 (1)	-1 (1)	6 (1)	-1 (1)
C (3)	15 (1)	21 (1)	17 (1)	-2 (1)	3 (1)	-1 (1)
C (4)	16 (1)	14 (1)	16 (1)	-1 (1)	4 (1)	0 (1)
C (5)	18 (1)	21 (1)	15 (1)	-1 (1)	4 (1)	2 (1)
C (6)	19 (1)	19 (1)	19 (1)	4 (1)	7 (1)	2 (1)
C (7)	17 (1)	18 (1)	20 (1)	2 (1)	7 (1)	0 (1)
C (8)	14 (1)	17 (1)	17 (1)	-1 (1)	3 (1)	0 (1)
C (9)	15 (1)	12 (1)	17 (1)	-2 (1)	4 (1)	0 (1)
C (10)	21 (1)	14 (1)	20 (1)	1 (1)	7 (1)	0 (1)
C (11)	16 (1)	22 (1)	19 (1)	0 (1)	2 (1)	3 (1)

6.6 *cis*-(7*S*,8*R*)-2-cyano-7-hydroxy-5,6,7,8-tetrahydroquinolin-8-yl 4-iodobenzoate (**33**)

1228mm C₁₇H₁₃IN₂O₃

6.6.1 Crystal data and structure refinement for compound **33**

Identification code	1228m
Empirical formula	C ₁₇ H ₁₃ I N ₂ O ₃
Formula weight	420.19
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system, space group	Orthorhombic, P2(1)2(1)2(1)
Unit cell dimensions	a = 8.7708(8) Å alpha = 90
deg.	b = 10.0936(10) Å beta =
90 deg.	c = 17.6552(17) Å gamma =
90 deg.	
Volume	1563.0(3) Å ³
Z, Calculated density	4, 1.786 Mg/m ³
Absorption coefficient	2.065 mm ⁻¹
F(000)	824

Crystal size	0.56 x 0.38 x 0.30 mm
Diffraction circle (sealed X-ray)	Bruker SMART APEX CCD 3-
graphite monochromator	tube, Mo Kalfa rad.,
512x512 pixels)	detector.distance 44.5 mm,
Scan type / width / speed	w-scan frames / dw = 0.30 /
10 sec. per frame	full sphere data collection,
4 x 600 frames	
Theta range for data collection	2.32 to 30.00 deg.
Index ranges	-12<=h<=12, -14<=k<=14, -
24<=l<=24	
Reflections collected / unique	28285 / 4551 [R(int) =
0.0268]	
Completeness to theta = 30.00	99.6%
Absorption correction	Multi-scan (program SADABS)
Max. and min. transmission	0.54 and 0.40
Structure solution	Direct methods (program
SHELXS97)	
Refinement method	Full-matrix least-squares on
F2 (prg SHELXL97)	
Data / restraints / parameters	4551 / 0 / 208
Goodness-of-fit on F2	1.078
Final R indices [I>2sigma(I)]	R1 = 0.0235, wR2 = 0.0572
R indices (all data)	R1 = 0.0244, wR2 = 0.0577
Absolute structure parameter	-0.009(13)
Largest diff. peak and hole	1.056 and -0.278 eA-3

$$R1 = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}, \quad wR2 = \left[\frac{\sum (w(F_o^2 - F_c^2))^2}{\sum (w(F_o^2))^2} \right]^{1/2}$$

6.6.2 Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 33

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

x	y	z	U_{eq}
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I (1)	1509 (1)	8914 (1)	5894 (1)	31 (1)
O (1)	2218 (2)	1424 (2)	3336 (1)	23 (1)
O (2)	3919 (2)	2881 (1)	4358 (1)	19 (1)
O (3)	5136 (2)	2720 (2)	5479 (1)	22 (1)
N (1)	6676 (2)	2580 (2)	3607 (1)	18 (1)
N (2)	9935 (3)	4343 (2)	3193 (2)	39 (1)
C (1)	7815 (2)	2563 (2)	3099 (1)	22 (1)
C (2)	7973 (2)	1623 (2)	2535 (1)	25 (1)
C (3)	6881 (2)	638 (2)	2482 (1)	24 (1)
C (4)	5683 (2)	614 (2)	2998 (1)	19 (1)
C (5)	4483 (2)	-449 (2)	2940 (1)	23 (1)
C (6)	3512 (3)	-576 (2)	3654 (1)	23 (1)
C (7)	3020 (2)	776 (2)	3929 (1)	20 (1)
C (8)	4423 (2)	1561 (2)	4164 (1)	17 (1)
C (9)	5634 (2)	1614 (2)	3549 (1)	17 (1)
C (10)	8972 (2)	3582 (2)	3169 (1)	27 (1)
C (11)	4293 (2)	3310 (2)	5056 (1)	17 (1)
C (12)	3566 (2)	4600 (2)	5245 (1)	17 (1)
C (13)	2453 (2)	5170 (2)	4778 (1)	19 (1)
C (14)	1851 (2)	6400 (2)	4967 (1)	22 (1)
C (15)	2360 (2)	7044 (2)	5617 (1)	20 (1)
C (16)	3441 (2)	6466 (2)	6091 (1)	20 (1)
C (17)	4043 (2)	5237 (2)	5903 (1)	18 (1)
H (10)	1459	1822	3516	35
H (2A)	8808	1653	2193	30
H (3A)	6950	-16	2096	28
H (5A)	4987	-1309	2839	28
H (5B)	3809	-250	2505	28
H (6A)	2601	-1122	3544	28
H (6B)	4107	-1026	4055	28
H (7A)	2329	671	4376	24
H (8A)	4874	1139	4625	21
H (13A)	2113	4720	4337	23
H (14A)	1098	6799	4654	26
H (16A)	3764	6908	6539	24
H (17A)	4778	4832	6223	22

Hydrogen atoms inserted in idealized positions

6.6.3 Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) compound 33

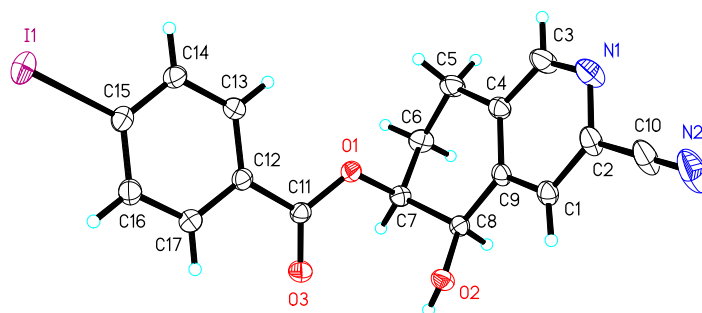
The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
I (1) 15 (1)	42 (1)	26 (1)	26 (1)	-3 (1)	-4 (1)	
O (1) 2 (1)	16 (1)	31 (1)	23 (1)	-3 (1)	-2 (1)	
O (2) 3 (1)	18 (1)	20 (1)	17 (1)	-3 (1)	-1 (1)	
O (3) 2 (1)	22 (1)	24 (1)	21 (1)	-2 (1)	-6 (1)	
N (1) 1 (1)	16 (1)	17 (1)	21 (1)	3 (1)	-1 (1)	
N (2) 8 (1)	29 (1)	38 (1)	50 (1)	16 (1)	-2 (1)	-
C (1) 2 (1)	15 (1)	19 (1)	30 (1)	7 (1)	1 (1)	
C (2) 6 (1)	20 (1)	28 (1)	26 (1)	5 (1)	7 (1)	
C (3) 6 (1)	25 (1)	25 (1)	20 (1)	-1 (1)	4 (1)	
C (4) 1 (1)	17 (1)	21 (1)	21 (1)	1 (1)	-1 (1)	
C (5) 3 (1)	22 (1)	23 (1)	25 (1)	-4 (1)	1 (1)	-
C (6) 5 (1)	22 (1)	22 (1)	26 (1)	-3 (1)	1 (1)	-
C (7) 2 (1)	16 (1)	26 (1)	18 (1)	0 (1)	0 (1)	-
C (8) 1 (1)	17 (1)	19 (1)	17 (1)	-1 (1)	0 (1)	
C (9) 1 (1)	13 (1)	20 (1)	17 (1)	2 (1)	-1 (1)	
C (10) 1 (1)	21 (1)	24 (1)	36 (1)	10 (1)	3 (1)	
C (11) 3 (1)	15 (1)	20 (1)	17 (1)	-1 (1)	1 (1)	-

C (12)	15 (1)	20 (1)	17 (1)	0 (1)	2 (1)	
0 (1)						
C (13)	17 (1)	25 (1)	16 (1)	0 (1)	0 (1)	
1 (1)						
C (14)	20 (1)	23 (1)	21 (1)	2 (1)	-2 (1)	
3 (1)						
C (15)	19 (1)	22 (1)	20 (1)	1 (1)	2 (1)	
2 (1)						
C (16)	20 (1)	21 (1)	19 (1)	-2 (1)	-2 (1)	-
1 (1)						
C (17)	16 (1)	23 (1)	17 (1)	2 (1)	-3 (1)	
0 (1)						

6.7 *cis*-(5*R*,6*S*)-3-cyano-5-hydroxy-5,6,7,8-tetrahydroisoquinolin-6-yl-4-iodobenzoate (**36**)



1239mm C₁₇H₁₃IN₂O₃

6.7.1 Crystal data and structure refinement for compound **36**

Identification code	1239xbm
Empirical formula	C ₁₇ H ₁₃ I N ₂ O ₃
Formula weight	420.19
Temperature	297 (2) K
Wavelength	0.71073 Å
Crystal system, space group	Orthorhombic, P2 (1) 2 (1) 2 (1)

Unit cell dimensions	a = 5.1072(3) Å	alpha = 90
deg.	b = 17.6739(11) Å	beta =
90 deg.	c = 18.1353(11) Å	gamma =
90 deg.		
Volume	1636.97(17) Å ³	
Z, Calculated density	4, 1.705 Mg/m ³	
Absorption coefficient	1.972 mm ⁻¹	
F(000)	824	
Crystal size	0.47 x 0.07 x 0.06 mm,	
colorless prism		
Diffractometer	Bruker SMART APEX CCD 3-	
circle (sealed X-ray	tube, Mo Kalfa rad.,	
graphite monochromator	detector.distance 50 mm,	
512x512 pixels)		
Scan type / width / speed	w-scan frames / dw = 0.3° /	
20 sec. per frame	3/4 sphere data collection,	
3 x 600 frames		
Theta range for data collection	2.25 to 27.04 deg.	
Index ranges	-6<=h<=6, -22<=k<=22, -	
20<=l<=23		
Reflections collected / unique	14336 / 3580 [R(int) =	
0.0335]		
Completeness to theta = 27.04	99.9%	
Absorption correction	Multi-scan (program SADABS;	
Sheldrick, 1996)		
Max. and min. transmission	0.89 and 0.62	
Structure solution	Direct methods (program	
SHELXS97)		
Refinement method	Full-matrix least-squares on	
F2 (prg SHELXL97)		
Data / restraints / parameters	3580 / 61 / 211	
Goodness-of-fit on F2	1.026	
Final R indices [I>2sigma(I)]	R1 = 0.0362, wR2 = 0.0765	
(2102 data)		
R indices (all data)	R1 = 0.0505, wR2 = 0.0822	
(3580 data)		
Absolute structure parameter	-0.02(3)	
Largest diff. peak and hole	0.81 and -0.27 eÅ ⁻³	

$$R1 = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}, \quad wR2 = \left[\frac{\sum (w(F_o^2 - F_c^2))^2}{\sum (w(F_o^2))^2} \right]^{1/2}$$

6.7.2 Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound 36

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U_{eq}
I (1)	1294 (1)	6708 (1)	4839 (1)	62 (1)
O (1)	10377 (5)	3950 (2)	5944 (2)	46 (1)
O (2)	10746 (5)	2414 (2)	5409 (1)	48 (1)
O (3)	11017 (7)	3897 (2)	4718 (2)	60 (1)
N (1)	7288 (9)	1980 (3)	7936 (2)	74 (1)
N (2)	4272 (14)	414 (3)	7282 (3)	109 (2)
C (1)	8758 (9)	1789 (2)	6684 (2)	48 (1)
C (2)	7276 (10)	1592 (3)	7296 (3)	60 (1)
C (3)	8811 (12)	2588 (3)	7948 (3)	68 (1)
C (4)	10332 (9)	2847 (3)	7366 (2)	50 (1)
C (5)	11799 (11)	3575 (3)	7439 (3)	62 (1)
C (6)	13757 (11)	3708 (3)	6816 (2)	57 (1)
C (7)	12685 (8)	3487 (2)	6076 (3)	45 (1)
C (8)	11979 (8)	2649 (2)	6073 (2)	41 (1)
C (9)	10303 (9)	2430 (2)	6715 (2)	43 (1)
C (10)	5604 (12)	934 (3)	7287 (3)	78 (2)
C (11)	9915 (8)	4159 (2)	5248 (2)	44 (1)
C (12)	7808 (8)	4740 (2)	5181 (3)	44 (1)
C (13)	6982 (8)	5173 (2)	5772 (3)	52 (1)
C (14)	5101 (9)	5732 (2)	5676 (3)	54 (1)
C (15)	4081 (7)	5845 (2)	4980 (2)	47 (1)
C (16)	4859 (9)	5415 (2)	4389 (3)	54 (1)
C (17)	6764 (9)	4876 (2)	4489 (3)	53 (1)
H (2O)	11790 (80)	2440 (30)	5080 (19)	72
H (1A)	8712	1494	6260	58
H (3A)	8863	2865	8383	82
H (5A)	12728	3576	7905	74
H (5B)	10555	3990	7448	74
H (6A)	14235	4239	6807	68

H (6B)	15332	3418	6914	68
H (7A)	13997	3588	5694	54
H (8A)	13620	2365	6115	49
H (13A)	7691	5088	6237	62
H (14A)	4541	6024	6073	65
H (16A)	4107	5488	3928	65
H (17A)	7359	4599	4086	63

Hydrogen atoms inserted in idealized positions

6.7.3 Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for compound **36**

The anisotropic displacement factor exponent takes the form:

$$-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
I (1)	48 (1)	45 (1)	94 (1)	11 (1)	2 (1)	2 (1)
O (1)	49 (2)	42 (1)	47 (1)	2 (1)	7 (1)	4 (1)
O (2)	54 (2)	53 (2)	38 (1)	-2 (1)	-2 (1)	-8 (1)
O (3)	66 (2)	64 (2)	50 (2)	3 (1)	18 (2)	16 (2)
N (1)	80 (3)	92 (3)	49 (2)	7 (2)	4 (2)	-22 (2)
N (2)	141 (6)	112 (4)	73 (3)	34 (3)	-25 (4)	-71 (4)
C (1)	53 (2)	49 (2)	43 (2)	6 (2)	-11 (2)	-3 (2)
C (2)	68 (3)	63 (3)	50 (2)	16 (2)	-9 (2)	-13 (2)
C (3)	83 (4)	81 (3)	41 (2)	-2 (2)	2 (2)	-15 (3)
C (4)	55 (2)	54 (2)	41 (2)	1 (2)	-5 (2)	-1 (2)
C (5)	79 (4)	61 (2)	45 (2)	-10 (2)	-2 (2)	-10 (2)
C (6)	51 (2)	62 (2)	58 (2)	-8 (2)	-6 (2)	-12 (2)
C (7)	41 (2)	47 (2)	47 (2)	-1 (2)	4 (2)	-3 (1)
C (8)	41 (2)	42 (2)	41 (2)	0 (2)	-5 (1)	3 (1)
C (9)	47 (2)	45 (2)	38 (2)	7 (1)	-8 (2)	1 (2)
C (10)	95 (4)	87 (3)	51 (3)	25 (2)	-14 (3)	-35 (3)
C (11)	48 (2)	38 (2)	46 (2)	-2 (2)	5 (2)	-3 (1)
C (12)	47 (2)	38 (2)	48 (2)	3 (2)	8 (2)	-1 (1)
C (13)	59 (3)	53 (2)	44 (2)	4 (2)	7 (2)	6 (2)
C (14)	57 (2)	50 (2)	55 (2)	1 (2)	12 (2)	9 (2)
C (15)	40 (2)	41 (2)	60 (2)	8 (2)	10 (2)	-2 (1)
C (16)	59 (2)	50 (2)	54 (2)	5 (2)	-2 (2)	1 (2)
C (17)	62 (3)	46 (2)	50 (2)	-2 (2)	0 (2)	3 (2)

7 Appendix

7.1 Publications resulting from this thesis

Poster Hungary

Muhammad Farooq Zia, Agnes G. Vasko, Zsuzsanna Riedl, György Hayos, and Marko D. Mihovilovic* Dihydroxylation of Quinolines and Isoquinolines by Recombinant Whole-Cell Biotransformation XII Blue Danube Symposium on Heterocyclic Chemistry, 10 -13 June 2007, Tihany, Hungary.

Poster Gordon conference

Muhammad Farooq Zia,^a and Marko D. Mihovilovic*^a Dihydroxylation of Nitrile Substituted N-Heterocycles by Recombinant Whole-Cell Biotransformation 2010.

Poster conference Wien

Muhammad Farooq Zia, Agnes G. Vasko, Zsuzsanna Riedl, György Hayos, and Marko D. Mihovilovic, Dihydroxylation of Nitrile Substituted N-Heterocycles by Recombinant Whole-Cell Biotransformation. 13th Austrian Chemistry Days, 24-27 August, 2009, Vienna University Of Technology, Vienna, Austria.

7.2 Curriculum vitae

Personal data

Name: Muhammad Farooq Zia
Date of birth: 08.01.1980
Family status: Married, 3 Children
Nationality: Pakistan
Age: 35



Address

Siegesplatz 1-3/1/9
1220, WIEN
AUSTRIA.
+43 699 181 39881
Email: ziafarooq@gmail.com

Education

1992 - 1994: Govt. comprehensive H/S school, Lahore (Matric Science)
1994 - 1996: Forman Christian College, Lahore (F. Sc. Pre-Medical)
1997 - 1999: Forman Christian College, Lahore (B. Sc. Botany, Zoology and Chemistry)
1999-2001: University of Punjab, Lahore (M. Sc. Chemistry) (Master Thesis: Physicochemical characterization of essential oil of *Ocimum basilicum*)
2004-2005: H.E.J. Research Institute of Chemistry, University of Karachi. (Ph. D. student in the field of Natural Products Chemistry)
2006- : Ph. D. studies-Institute of Applied Synthetic Chemistry, Vienna University of Technology (Supervisor Univ. Prof. DI. Dr. Marko D. Mihovilovic).

Other professional experience

Actively involved as assistant in organization of 9th FIGIPAS Meeting in Inorganic Chemistry 4-7 Juli 2007, Vienna, Austria.
Actively involved as assistant in organization of Biotrans 2015, Juli 26-30 2015, Vienna, Austria.

IT skills: MS Windows 7/8/10, Mac OS, MS Office, Beilstein Crossfire, Scifinder, Reaxys, Isis Draw, WinNMR, MestreNOVA, Networking, and Virtualization.

Other relevant skills: GC-, GC/MS- and HPLC-operator in the research group of Prof. Marko D. Mihovilovic and Prof. Peter Stanetty (Institute of Applied Synthetic Chemistry, Vienna University of Technology).

Publication activity

3 posters

Languages

English, Urdu, German (Intermediate)

7.3 List of abbreviations

ACN	acetonitrile
Amp	Ampicillin
NPAH	aza-polycyclic aromatic hydrocarbons
b.p.	boiling point
BVMO	Baeyer-Villiger monooxygenase
DO	dioxygenase
DD	diol-dehydrogenase
BDO	benzene dioxygenase
BZDO	benzoate dioxygenase
NDO	naphthalene dioxygenase
TDO	toluene dioxygenase
BPDO	biphenyl dioxygenase
CDO	chlorobenzene dioxygenase
Dioxane	1,4-dioxane
DCM	dichloromethane
DMSO	dimethylsulfoxide
EH	epoxide hydrolase
EtOAc	ethyl acetate
Et ₂ O	diethyl ether
EtOH	ethanol
FAD	flavin adenine dinucleotide
HPLC	high pressure liquid chromatography
IPTG	isopropyl- β -thiogalactopyranoside
Kan	kanamycin
LP	light petroleum (b.p. 40-60°C)
m.p.	melting point
MeOH	methanol
OD	Optical Density
PAH	polycyclic aromatic hydrocarbons
Py	pyridine
R _f	ratio to the front for TLC
Rpm	revolutions per minute

Rt	room temperature, 25 °C
THF	tetrahydrofuran
NEt ₃	triethylamine
MPLC	Medium Pressure Liquid Chromatography
TLC	thin layer chromatography

7.4 Elemental analysis reports

Reference No. 0308/0173

Sample ID	Anal Nr	w-% C	w-% H	w-% N	Compound
ZF 70	5.3/39	62.61	4.19	14.71	15
ZF 92	5.3/40	62.86	5.33	14.38	25
ZF 104	5.3/41	44.17	2.43	4.28	32
ZF 110	5.3/42	63.94	5.62	13.92	21
ZF 119	5.3/43	60.88	3.60	12.94	39

Reference No. 0706/0684

Sample ID	Anal Nr	w-% C	w-% H	w-% N	
FZ 036 F2	11.7/26	63.65	4.24	14.61	10

Reference No. 0308/0173

Sample ID	Anal Nr	w-% C	w-% H	w-% N	
ZF 070 EE	3.6/37	63.77	3.95	14.76	15
	3.6/53	63.88	4.13	14.76	
ZF 104 F2	6.6/27	48.13	3.05	6.17	33
	6.6/55	48.20	3.02	6.20	
ZF 107 F1	3.6/23	49.00	3.86	4.04	34
ZF 107 F2 C-1	3.6/24	45.01	2.53	4.04	35
	6.6/28	44.89	2.5	4.16	
ZF 107 F2 C-2	6.6/29	44.28	2.24	4.19	34
FZ 107 F3 A-M	3.6/27	48.92	3.01	6.43	33
ZF 107 F3 C-2	3.6/26	48.36	2.80	6.49	36
ZF 110 A	3.6/28	63.90	5.28	14.08	21

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