FACULTY OF BIOSCIENCE ENGINEERING

CHEMICAL SYNTHESIS AND IMINE REDUCTASE-CATALYZED ENANTIOSELECTIVE REDUCTION OF TRIFLUORINATED IMINES

Helder Coltura
Student number: 01508605

Promotors: Prof. dr. ir. Matthias D'hooghe and Prof. dr. Tom Desmet
Tutors: ir. Sari Deketelaere and ir. Shari Dhaene

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in Bioscience Engineering: Chemistry and Bioprocess Technology

Academic year: 2019 - 2020

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Voorwoord

De voorbije vijf jaar aan de faculteit Bio-ingenieurswetenschappen zijn voorbij gevlogen. Tijdens deze periode heb ik de kans gekregen om bij te leren over allerlei takken van de wetenschap en technologie. De academische interesses en passies die ik hier heb ontwikkeld zullen mij ongetwijfeld levenslang bijblijven. Deze thesis vormt een passend sluitstuk voor mijn opleiding als bio-ingenieur. Uiteraard had ik dit werk niet kunnen afronden zonder de hulp van een aantal personen.

Allereerst zou ik graag mijn promotors, professor Matthias D'hoohge en professor Tom Desmet willen bedanken om mij de kans te geven dit boeiende onderzoek uit te voeren. Jullie vakken organische chemie, en biokatalyse en enzymtechnologie en de manier waarop jullie lesgaven, hebben op mij een enorme indruk achtergelaten. Om deze reden heb ik zonder aarzelen voor dit gedeelde onderwerp gekozen. Ik ben in het bijzonder gebeten door de organische chemie en kan het tot op de dag van vandaag niet laten om af en toe een handboek open te slaan om alle geheimen van dit veld te ontrafelen.

Mijn begeleiders, Sari en Shari, bedankt voor jullie goede begeleiding tijdens dit project. Op momenten dat ik gedemotiveerd of bezorgd was, waren jullie er steeds om mij gerust te stellen. Jullie hielpen mij om structuur te scheppen in mijn chaos en stuurden bij wanneer ik afdwaalde. Al jullie nalezen heeft enorm geholpen om hier een mooi werk van te maken.

Ook wil ik al mijn medethesisstudenten bedanken voor de gezellige sfeer en voor de nodige *banter* tijdens de koffiepauzes. Ook een oprechte dankjewel aan alle doctoraatstudenten in beide labo's om al mijn vragen te beantwoorden en advies te geven.

Ik wil ook mijn vrienden, de oude van de noordrand en de nieuwe van de bio-ingenieurs, bedanken voor alle leuke momenten de voorbije jaren. Moge er nog veel van die momenten komen in de toekomst.

Maïa, bedankt om er steeds voor mij te zijn en in mij te geloven het voorbije jaar. Je was een luisterend oor en je stond altijd voor me klaar om me op te beuren als het moeilijk ging. Ook je structuur- en overzicht*skills* hebben mij echt geholpen bij het schrijven van dit werk. Verder wil ik je ook bedankten voor de leuke jaren die we al samen hebben doorgebracht.

Mama en Dirk, papa en Ariane, bedankt voor al jullie steun de voorbije jaren en om steeds in mij te geloven, zelfs wanneer anderen of ikzelf dat niet deden. Luca en Celien, bedankt voor het nalezen en dankjewel om zo'n lieve broer en zus te zijn.

Helder Coltura, 21 augustus 2020

Preamble

The experimental work for this Master's thesis was planned for the period of August 2019 to May 2020. However, in the course of the second semester, a pandemic outbreak of the SARS-Cov-2 virus resulted in an early termination of all lab activities as of March 19th. As a result, some planned experiments could not be performed. These include

- the optimization of the enzymatic reactions in terms of cosolvent, substrate concentration, reaction time, pH, etc.;
- the determination of the enantiomeric excess of the enzymatic reactions;
- performing preparative enzymatic reactions and subsequent derivatization of the products;
- the determination of the absolute configuration of the enzymatically formed products.

Even so, it was deemed by the author, the tutors and the promotors that sufficient data had been collected to draw some interesting conclusions and to finalize this thesis. In the discussion of the results (section 3), any gaps in the experimental data were complemented with literature insights, whenever possible. Furthermore, additional experiments were proposed for future research.

This preamble was drawn up after consultation between the student and the promotors and is approved by all three.

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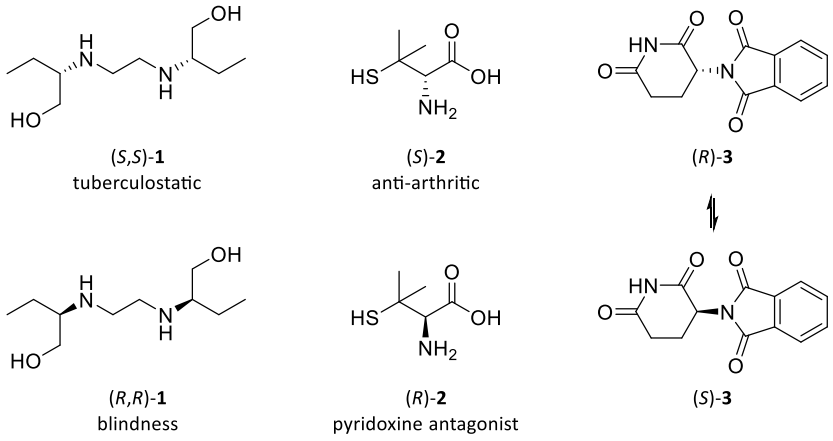
“It is the mark of an educated mind to be able to entertain a thought without accepting it.”

Aristotle

1 Background and goal

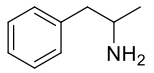
1.1 Background

Chiral organic molecules play an exceedingly important role in modern life, especially when biological activity is concerned, like in pharmaceuticals and – to a lesser extent – agrochemicals. Chiral molecules are characterized by the fact that their mirror images are non-superimposable, similar to a person's right and left hand. These two possible mirror images of a chiral molecule are called enantiomers. Enantiomeric molecules are identical in most physical properties like density, melting point, boiling point, etc., but may differ significantly in their biological activity. This can be explained by the fact that the biomolecules (enzymes, receptors, etc.) involved in the biological activity are themselves chiral and exist in nature as single mirror images. Therefore the two enantiomers of the bioactive compound will likely interact differently with these biomolecules.[1] For instance, the (*S*,*S*)-enantiomer of ethambutol **1** is used to treat tuberculosis, while the (*R*,*R*)-enantiomer causes blindness.[2] Similarly, (*S*)-penicillamine (*S*)-**2** has anti-arthritic properties, while the corresponding (*R*)-enantiomer is a pyridoxine antagonist and thus toxic.[3] However, the most notorious example is without a doubt thalidomide **3**. In the early 1960s, this drug was used as a racemic mixture for the treatment of morning sickness in pregnant women. This resulted in an international tragedy in which more than 10 000 babies were born with horrible birth defects.[4,5] The teratogenic effects of thalidomide have been ascribed to the (*S*)-enantiomer,[6,7] although this has been disputed.[8] Whatever the case may be, this is not a very meaningful discussion since thalidomide racemizes rapidly *in vivo*.[9] However, for most chiral drugs, the unwanted side effects of one of the enantiomers can be avoided by administering only the good enantiomer. Accordingly, there has been a lot of interest in the development of preparation methods for enantiopure compounds.



Chiral amines, and more specifically α-chiral amines, are some of the most important building blocks in a broad range of pharmaceuticals, agrochemicals and other fine chemicals.[10–12] An estimated 40% of all pharmaceuticals contain at least one chiral amine moiety.[13] Chiral amines are also ubiquitous in the biological world, for example in amino acids and alkaloids.

Amphetamine derivatives constitute a diverse and important subclass of chiral amines with a variety of medicinal applications. They are based on the amphetamine **4** structure, which is characterized by a basic nitrogen, separated from an aromatic ring by two carbon atoms and an α-methyl group.[14,15] One of the most notable characteristics of this class of molecules is that small changes in their structure may result in vastly different biological activities, ranging from stimulation to sedation to hallucinogenic effects. Amphetamine derivatives interact with the biological targets and receptors of the mono-amine neurotransmitters: norepinephrine **5**, epinephrine **6**, dopamine **7** and serotonin **8**.[16] Naturally occurring amphetamine derivatives include cathinone **9** and ephedrine **10**.[14,17] Important pharmaceutical examples include bupropion **11**[18] (antidepressant) and selegiline **12**[19] (treatment of Parkinson's disease). The latter is sold as a single (*R*)-enantiomer.[19]



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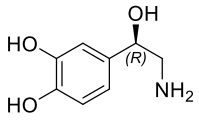

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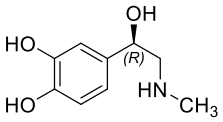

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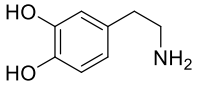

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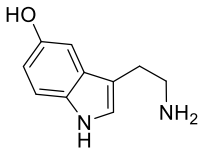

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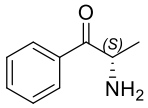

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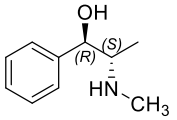

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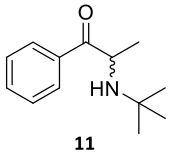

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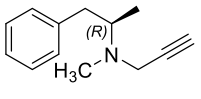

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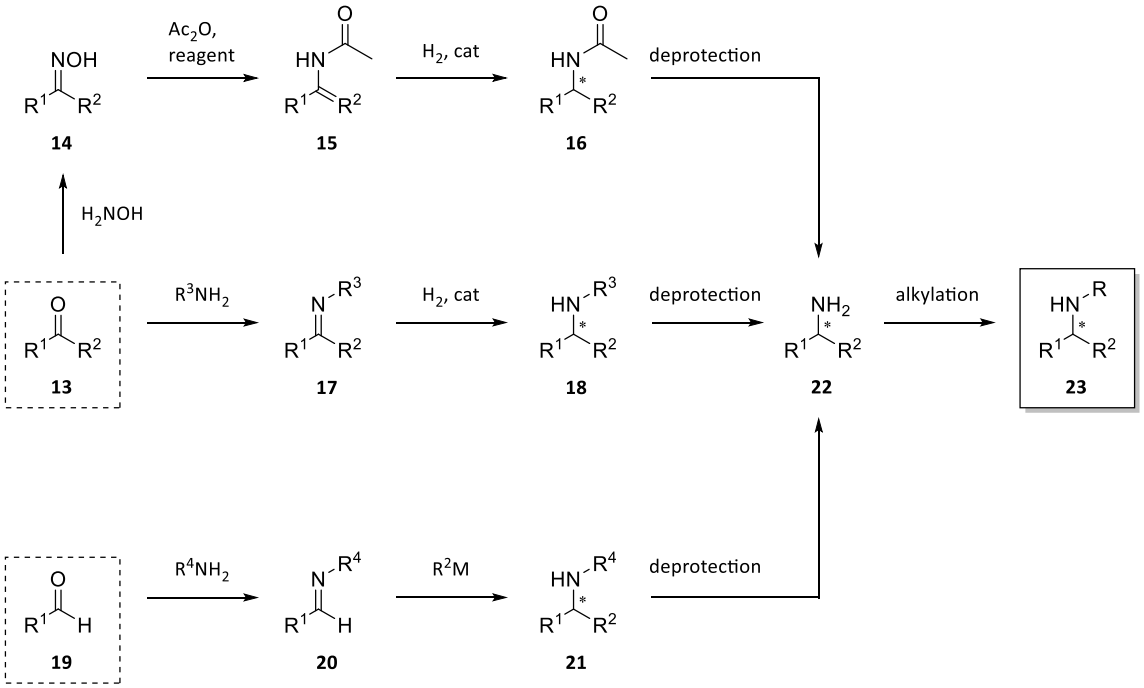
Classical organic synthesis methods for chiral amines without elements of selectivity usually result in racemic mixtures. However, multiple methods have been developed to prepare optically pure amines.

Resolution methods involve the separation of a mixture of amine enantiomers. This can be achieved by adding an enantiomerically pure carboxylic acid to the amine racemate, with subsequent formation of a pair of diastereomeric salts that are separable through crystallization, *i.e.* classical resolution. There is also a variant called kinetic resolution, which employs a chiral catalyst (a chemical or an enzyme) that discriminates between the enantiomers of a racemate

and transforms one with a higher rate, thus leaving the other one (mostly) unchanged. These methods remain important in industry, despite the fact that the yield is limited to 50%.[10,20,21]

A second industrially relevant route toward optically pure amines is the enantioselective transformation of imines **17**, **20** or enamides **15** derived from ketones **13** and aldehydes **19**.[12] The two main methods are the transition metal-catalyzed hydrogenation of ketimines **17** and enamides **15**,[22–24] and the addition of carbanions to aldimines **20**.[25,26] The stereoselectivity usually arises either from a chiral ligand-metal complex or from a chiral auxiliary.[22–26]

These chemical processes are complicated by environmental (heavy metals) and safety (high pressure H_2 gas, flammable metal-organic reagents) hazards, as well as water- and air-sensitive reagents which are difficult to handle. They also require protection/deprotection steps for nitrogen activation and/or chiral induction as well as a possible alkylation step to yield the desired secondary amine **23**.[22–26] These extra steps are wasteful and lower the overall efficiency and sustainability of these chemical processes. Moreover, the use of organic solvents and rare precious metals also contributes to the unsustainability of these processes.[27,28]



Enzymatic reactions have the advantage that they are usually environmentally benign, safe and easy to handle. In general, enzymes operate in aqueous buffers and under mild conditions (T, p, pH), are produced from renewable biomass, are biodegradable and are non-toxic. Maybe even more important is their high regio- and chemoselectivity, which make protection/deprotection steps unnecessary and minimize side product formation, thus significantly improving the efficiency compared to classical processes. Lastly, the use of enzymes in pharmaceutical

processes eliminates the cost of removing transition metal traces from the products.[29] For these reasons, biocatalysis is considered a greener, more sustainable alternative to the classical organic synthesis methods for chiral amines.[30–33]

Of course, biocatalysis does have some limitations which prevent it from completely replacing classical organic synthesis. For instance, some products or substrates are not stable in water. Additionally, some enzymes or enzyme classes have a narrow substrate scope with regard to unnatural substrates or are unstable at operating conditions. Furthermore, enzymes may suffer from product or substrate inhibition, which imposes low substrate concentrations and therefore long reaction times and low productivities. Luckily, much improvement has been made on these issues using protein engineering techniques like directed evolution.[33,34]

Fluorine plays a central role in medicinal chemistry nowadays. In 2019, 38% of new FDA approved small molecule drugs contained at least one fluorine atom.[35] Fluorinated amines in particular constitute an important class of medicinal compounds. For example, β-fluorinated amines have been shown to be potent inhibitors of pyridoxal 5'-phosphate (PLP)-dependent enzymes.[36] Furthermore, α-trifluoromethyl amines have been used as metabolically stable isosteres for amide and peptide bonds on account of the isopolarity of C-CF_3 and C=O functionalities.[37–40] The presence of fluorine atoms in amines can alter their physical, chemical and biological properties substantially. Due to its ultimate electronegative character,[41] fluorine inductively withdraws electron density from the nitrogen atom, resulting in amines with lowered basicity.[42–44] These amines are therefore more likely to be present in neutral form at physiological pH, which can change *in vivo* interactions, improve oral bioavailability and enhance uptake through the blood-brain barrier (BBB).[44–46] This is especially interesting for amphetamine derivatives like selegiline **12**, since these molecules are often active in the central nervous system (CNS) and must therefore cross the BBB.[16,47] The potential of these trifluoromethyl amphetamine derivatives is largely unexplored in the literature.[48,49]

1.2 Goal

The aim of this Master's thesis is to enantioselectively synthesize trifluorinated amphetamine derivatives **26** and **27** by means of imine-reducing enzymes called imine reductases (IREDs). This preliminary, explorative research project will be conducted at the Department of Green Chemistry and Technology and the Centre for Synthetic Biology (Faculty of Bioscience Engineering).

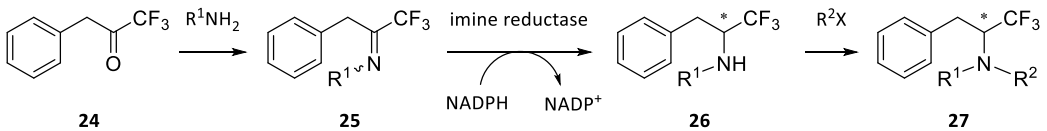
Trifluoromethyl derivatives of amphetamines are an interesting synthesis target for medicinal chemistry research. The trifluoromethyl group is known to lower the basicity of amines through

its inductive electron-withdrawing character. This can significantly influence their *in vivo* interactions, improve their oral bioavailability and enhance their uptake through the blood-brain barrier.[44–46] This class of amphetamine derivatives is largely unexplored, which provides an opportunity for research.[48,49] Furthermore, biocatalysis could provide a sustainable route to enantiopure trifluoromethyl amphetamines, which is an important requirement in pharmaceutical applications. Moreover, the enzymatic reduction of trifluoromethyl imines would constitute a completely novel method for the preparation enantiopure trifluoromethyl (amphet)amines.

In the first part of this project, trifluoromethyl benzyl ketimines **25** will be synthesized by condensation of 1,1,1-trifluoro-3-phenylpropan-2-one **24** with primary amines. Imines with a sterically undemanding *N*-substituent (*e.g. n*-propyl) will be attempted first, to ensure that an enzymatic reduction can be achieved in the screening of the IREDs. Hereafter, imines with more biologically interesting *N*-substituents (*e.g.* propargyl) will be attempted, with the aim of making CF_3 derivatives of pharmaceutical amphetamines like selegiline **12**.

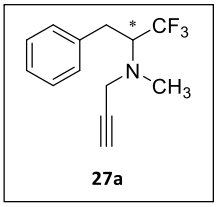
Next, two pairs of enantiocomplementary IREDs will be screened for reduction activity toward imines **25**, in hopes of accessing both enantiomers of secondary amines **26**. These amines **26** are trifluoromethyl amphetamine derivatives with potentially interesting biological activities. The product formation as well as the enantioselectivity of each IRED-catalyzed imine reduction will be assessed. After optimization of the reaction conditions, preparative scale reactions will be performed. The isolated optically enriched secondary amines **26** will be further derivatized as well as characterized in terms of their absolute configuration.

Finally, secondary amines **26** will be alkylated with alkyl halides with the intent of creating more trifluoromethyl amphetamine derivatives **27** like the selegiline derivative **27a**.



24 **25** **26** **27**

(R^1 = *n*Pr, propargyl)
(R^2 = Me)



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2 Literature review

2.1 Introduction

This chapter serves to introduce the reader to recent developments on the enzymatic production of chiral amines using imine reductases (IREDs). A brief overview is given of the main enzyme classes used for chiral amine production, followed by an in-depth discussion of IREDs. Structure, mechanism, stereoselectivity, cofactor regeneration, and a summary of the recent developments in the field will be discussed.

2.2 Overview of enzymatic approaches to chiral amines

In the last 30 years, multiple biocatalytic routes have been developed for the synthesis of chiral amines.[13] Lipases catalyze the formation and cleavage of amides (and esters) and have been used to perform kinetic resolutions of racemic amines. The lipase selectively acylates one enantiomer of the amine and leaves the other unreacted.[50] ω-transaminases (ω-TA) catalyze the pyridoxal phosphate (PLP)-dependent transfer of an amino group from a sacrificial amine donor to a carbonyl compound, essentially converting an aldehyde or ketone to a (chiral) primary amine.[51,52] Amine dehydrogenases (AmDH) catalyze the nicotinamide adenine dinucleotide (NADH)-dependent reductive amination of ketones with ammonia, generating a primary amine.[53] Amine oxidases (AO) catalyze oxidation of primary amines to imines with reduction of molecular oxygen to hydrogen peroxide and have been combined with non-selective chemical reducing agents for the deracemization of amines.[13,54,55]

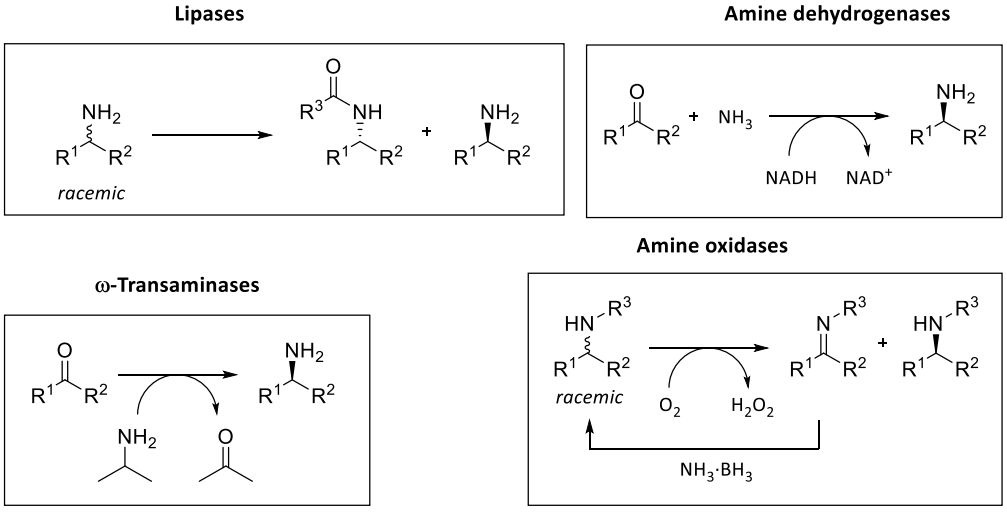


Figure 1. Overview of some of the main enzymatic approaches toward chiral amines.

Most of these enzyme classes only generate primary amines. A more recently discovered class of enzymes, called imine reductases (IRED), provides a route toward chiral primary, secondary and tertiary amines through reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent C=N reduction. IREDs can form amines either from preformed cyclic imines (imine reduction) or from exocyclic imines formed *in situ* by condensation of carbonyl compounds with amines (reductive amination). In the case of the latter, reductive aminases (RedAm), a special subclass of IREDs that can catalyze the imine formation in addition to the imine reduction, can be used.[56–58]

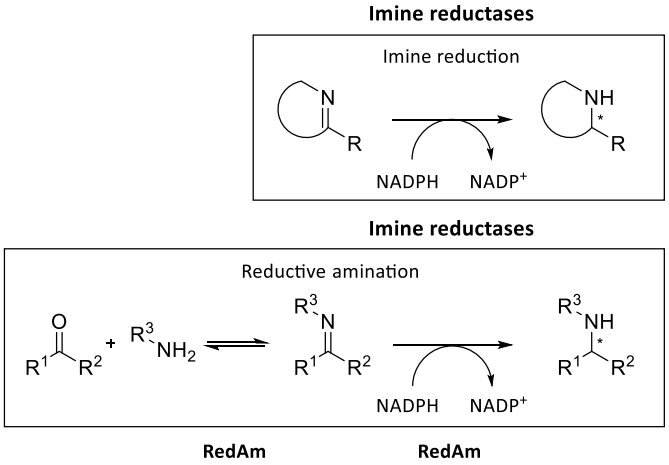
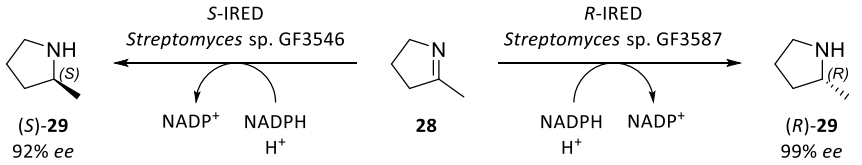


Figure 2. Imine reductase-catalyzed reactions.

2.3 Imine reductases (IREDs)

Imine reductases (IREDs) are nicotinamide-dependent enzymes that catalyze the asymmetric reduction of prochiral imines and iminium ions to the corresponding primary, secondary and tertiary amines.[56,57] This class of enzymes has seen a slow development compared to the analogous ketone reductases (KREDs), which have been applied in multiple industrial scale processes for the asymmetric synthesis of chiral alcohols.[32,59,60] The biocatalytic reduction of imines was long thought to be unfeasible due to the hydrolytic instability of imines, especially exocyclic imines in aqueous environments.[61–63] In the last decade, however, the interest in this field was rekindled with the discovery and characterization of two enantiocomplementary, NADPH-dependent IREDs by Mitsukura *et al.* These enzymes were discovered in *Streptomyces* sp. GF3587 (*R*-IRED-*Ss*) and *Streptomyces* sp. GF3546 (*S*-IRED-*Ss*) through classical microbial screening. After heterologous expression in *E. coli* and purification, these IREDs were able to reduce the hydrolytically stable cyclic imine 2-methyl-1-pyrroline (2-MPN) **28** to (*R*)- or (*S*)-2-methylpyrrolidine **29** with high enantioselectivities.[62,64–66] Imine **28** serves as a model substrate

for IREDs and is often used in the literature to characterize newly discovered IREDs in terms of their activity and *R*/*S* stereoselectivity.[56]



Following these initial IRED discoveries, several other enzymes in this family have been described. To facilitate the discovery of new productive IREDs, an Imine Reductase Engineering Database with putative IRED sequences was constructed using a BLAST (Basic Local Alignment Search Tool) search based on sequence similarity to the characterized enzymes *R*-IRED-*Sk*[67] and *S*-IRED-*Ss*.[65] The putative IRED sequences in this database were assigned to two superfamilies, the *R*-IRED type and the *S*-IRED type superfamily.[68] This database was expanded and the superfamily classification was refined, resulting in 14 superfamilies (SFam1-14). Most of the reported *R*- and *S*-selective IREDs belong to SFam1 and SFam2, respectively.[69] As of June 2020, the Imine Reductase Engineering Database contains 1409 putative IRED sequences, the vast majority of which are of bacterial origin.[70]

2.3.1 Structure and mechanism

2.3.1.1 General IREDs

The first enzyme with confirmed IRED activity toward 2-methyl-1-pyrroline **28** to be crystalized and structurally characterized using X-Ray is *R*-IRED-*Sk* or Q1EQE0 from *Streptomyces kanamyceticus* (Figure 3). This enzyme is composed of two intertwined monomers, which are bound by domain swapping. It has two active sites, each containing an $NADP^+$ molecule.[67] Other IRED crystal structures were subsequently reported and all had the same basic structure.[71–76] An IRED monomer consists of two domains: the N-terminal Rossmann fold domain and the C-terminal helical domain, which are connected by a long interdomain helix. The active site is a cleft formed at the interface between the N-terminal domain and the C-terminal domain of the two different monomers. The Rossmann fold is highly conserved in all IREDs and binds the NADPH cofactor. The helical C-terminal domain on the other hand, is quite variable. Furthermore, the substrate binding cleft is mainly hydrophobic.[69]

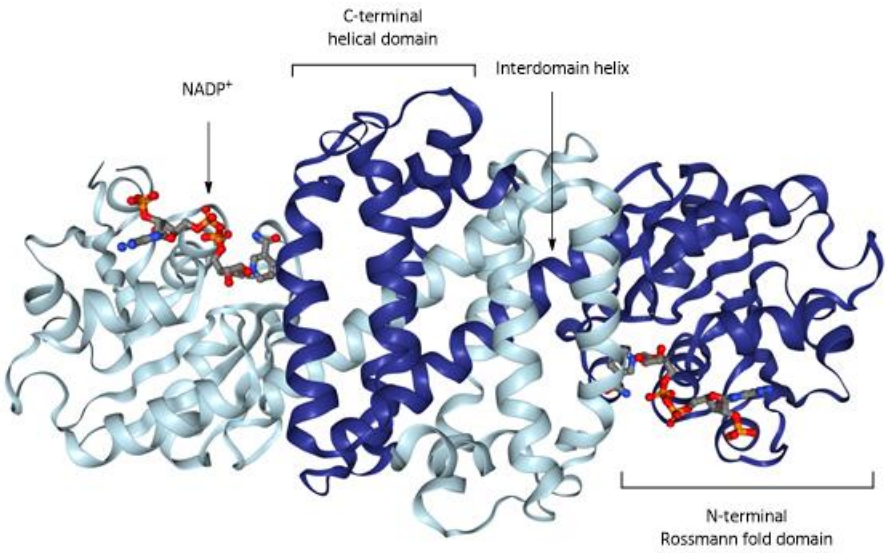
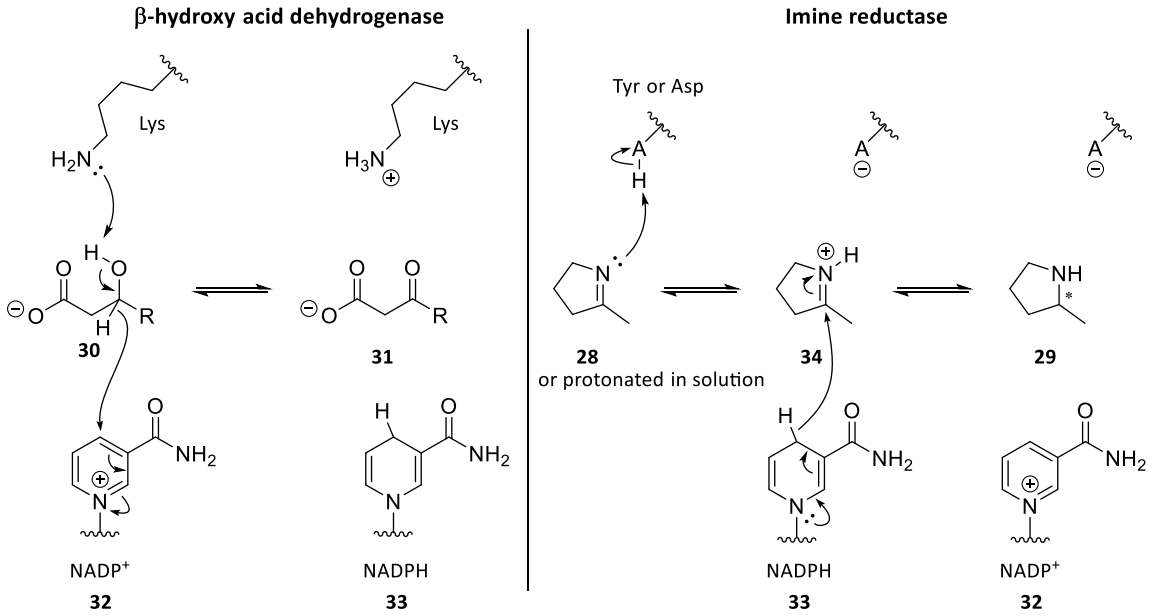


Figure 3. Cartoon representation of the crystal structure of the *R*-selective IRED from *Streptomyces kanamyceticus.* The two monomers have different colors. Image from the RCSB PDB (rcsb.org) of PDB ID 3ZHB.[67]

An imine reduction mechanism was first proposed for *R*-IRED-*Sk* based on a structural comparison with β-hydroxy acid dehydrogenases (β-HAD), a well-known enzyme class similar in structure and sequence to IREDs.[67,69] These enzymes catalyze the oxidation of an alcohol **30** where a proton is abstracted from the oxygen by a lysine residue and a hydride is transferred to $NADP^+$ **32**.[77] In *R*-IRED-*Sk* and in most other *R*-selective IREDs, an aspartic acid residue was found at the equivalent position (standard position 187) of the lysine.[67–69] For most *S*-selective IREDs, a tyrosine was found at this position.[68,71,72] In the proposed mechanism, this Asp or Tyr residue protonates imine **28**, after which NADPH **33** transfers a hydride to iminium species **34**.[67,68,72] This mechanism is supported by the fact that mutation of the Asp or Tyr in some IREDs to aprotic residues resulted in inactive enzymes.[67,68] However, some of these mutants retained some activity.[68] Moreover, active wild-type IREDs with aprotic residues such as alanine,[78] asparagine[73,79] or phenylalanine[79] at this position have been reported. These findings suggest a different mechanism, in which protonation by a catalytic residue is not essential. In fact, since imines are slightly basic, they might already be protonated in solution and enter the active site as such, depending on the pH.[56,69,75] The main role of IREDs would thus be to place NADPH and iminium species **34** in an optimal position for hydride delivery.[69] This theory is supported by the fact that *N*-alkyl iminium ions are reported as good substrates for IREDs.[74,80,81] However, reductive amination experiments at basic conditions (pH 9.3), where imines are not expected to be protonated in solution, resulted in good conversions, which is not consistent with the last theory.[82] It is clear that more research is required to determine the mechanism unequivocally.



For most IREDs, the stereochemical outcome is completely determined by the enzyme itself (for exceptions see section 3.2.2.3 Stereoselectivity).[71,79,80,82–86] A conservation analysis revealed that *R*- and *S*-selective IREDs can be grouped based on sequence similarity, with most *R*- and *S*-selective IREDs belonging to SFam1 and SFam2, respectively.[69] Initially the putative proton donor residue at standard position 187 was thought to be the main determinant for stereopreference as these are highly conserved within the superfamilies (Asp → *R*-IRED, Tyr → *S*-IRED).[69] However, exceptions to this rule have been reported, such as IR_10 from *Mycobacterium smegmatis*[79] and IRED-G from *Streptomyces rimosus* ATCC 10970[87] which have an Asp residue but are *S*-selective. Furthermore, some IREDs have neither tyrosine or aspartic acid at this position.[73,78] Analysis of active site residues revealed two more standard positions (139 and 194) which determine the stereochemistry. *S*-selective IREDs are characterized by a proline at position 139 and a phenylalanine at position 194. *R*-selective IREDs typically have a hydrophobic residue (valine or isoleucine) or a threonine at position 139 and a methionine or leucine at position 194.[69] Exceptions have been found for these rules as well, indicating that linking stereopreference to amino acid residues can be difficult.[88]

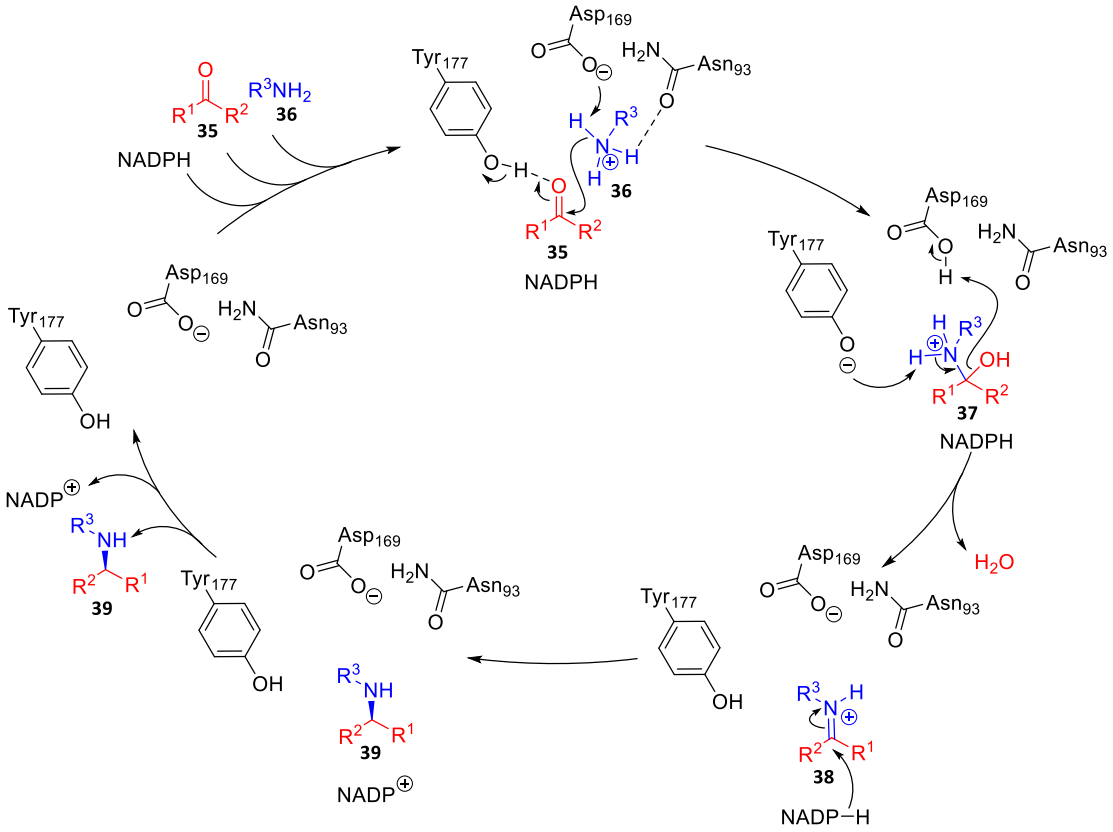
2.3.1.2 Reductive aminases

Reductive aminases or RedAms are a subclass of IREDs of fungal origin which catalyze imine formation from a carbonyl compound and an amine, on top of the usual imine reduction.[89] The first member of this subclass was an enzyme from *Aspergillus oryzae* (*Asp*RedAm). Other fungal RedAms sequences were discovered in *Aspergillus terreus* (*At*RedAm), *Ajellomyces dermatitidis* (*Ad*RedAm),[74] *Neosartorya fumigatus* (*Nf*RedAm) and *Neosartorya fischeri* (*Nfis*RedAm).[90] There

have also been some reports of bacterial IREDs for which reductive aminase activity has been suspected but not confirmed.[91,92]

By studying the kinetics of reductive aminations with *Asp*RedAm including product inhibition studies, the kinetic mechanism of this enzyme was elucidated. The reductive amination was shown to follow a Ter-Bi (three substrates, two products) ordered sequential mechanism in which NADPH, the ketone and the amine are combined with the enzyme in that order. This is followed by the sequential release of the amine product and $NADP^+$.[74]

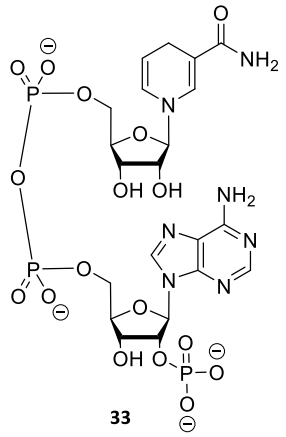
A catalytic mechanism for the imine formation inside the active site of these RedAms was also proposed based on crystallographic data and mutagenesis studies of *Asp*RedAm. First, ketone **35** is coordinated to a Tyr-177. Next, amine **36**, which is initially positively charged, is deprotonated by the carboxylate of Asp-169. Amine **36** subsequently attacks ketone **35** forming *N*-protonated hemiaminal **37**, which is dehydrated *via* two concerted proton exchanges by Asp-169 and the phenolate of Tyr-177. Finally, the formed iminium ion **38** is reduced to amine **39** by a hydride from NADPH.[89]



2.3.2 Practical considerations with IREDs

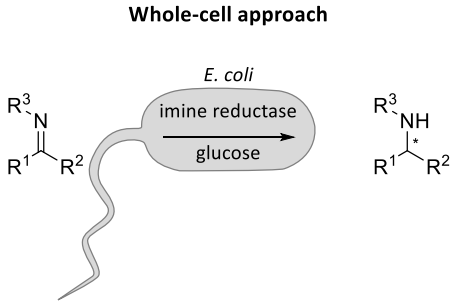
3.2.2.1 Cofactor regeneration

All of the described IREDs use NADPH **33** as a cofactor. In each reaction one imine molecule is reduced to the corresponding amine and one NADPH molecule is oxidized to $NADP^+$ *via* a hydride transfer.[67,68,72] However, NADPH is very expensive, *i.e.* 215 000 US$ per mole in bulk (2011), therefore it cannot be used stoichiometrically and must be regenerated *in situ* to ensure economic feasibility.[93] This can be achieved by performing the reaction in a whole-cell biotransformation, or by using a cofactor regeneration reaction.



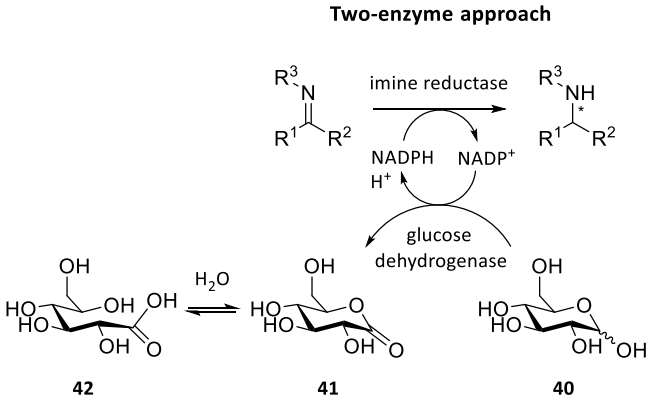
33

In a whole-cell biotransformation, the production enzyme is heterologously expressed in a host organism such as *E. coli*. The resting whole cells are then used to convert the substrate and the cofactor is regenerated by the natural cellular metabolism or by a co-expressed glucose dehydrogenase,[88] which requires only glucose. The main advantage of this method is that cofactor addition and enzyme purification are not required. However, the yield can be low due to metabolism of the substrate or product and difficult product recovery.[84,94–96] For industrial applications, the whole-cell approach is often preferred.[97]

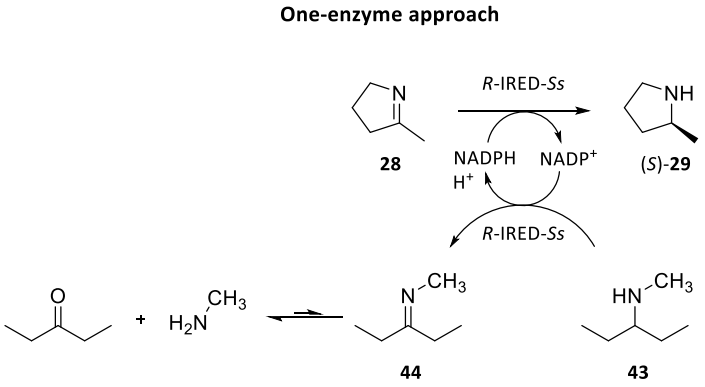


In the case of an *in vitro* set-up with purified enzymes or lysed cells, the most common cofactor regeneration method is to couple the imine reduction reaction to a second enzymatic reaction in which a cheap reductant like D-glucose **40** is oxidized by a second enzyme to reduce the $NADP^+$

to NADPH. The cheap reductant is added in excess, providing a thermodynamic driving force for product formation.[98] For IRED-catalyzed reactions, a glucose dehydrogenase (GDH) is often used in combination with D-glucose **40**, with the formation of D-glucono-δ-lactone **41**.[79,82,99] Subsequently, lactone **41** is hydrolyzed to D-gluconic acid **42**, which can impair the reaction if no pH control is used.[82] Other dehydrogenases such as glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*[67,72,83,84] or alcohol dehydrogenase from *Lactobacillus brevis* in combination with isopropanol[87] are also reported.



A second *in vitro* cofactor regeneration strategy is a one-enzyme approach with a sacrificial amine co-substrate which is oxidized to regenerate NADPH. In this approach, the IRED catalyzes both the product forming reduction reaction and the regeneration reaction. The amine co-substrate (*e.g.* **43**) is oxidized to an acyclic or exocyclic imine (*e.g.* **44**), which is subsequently hydrolyzed to the corresponding ketone and amine. This method was demonstrated for the reduction of 2-methyl-1-pyrroline **28** with *R*-IRED-*Ss* with the sacrificial amine **43** and up to 60% conversion was achieved. This approach is complicated by the fact that the optimum pH for imine reduction and amine oxidation differ and oxidation is typically slower than reduction.[100]



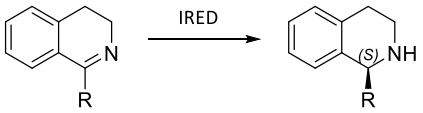
In industrial applications, NADH-dependent production enzymes are preferred over NADPH-dependent ones, due to the 70-fold price difference of NADPH over NADH (sevenfold for the

oxidized forms) and the abundancy of NADH regeneration systems.[93,100] To that end, there have been efforts to improve the NADH/NADPH specificity of IREDs using (semi-)rational protein engineering.[98,100] Recently, a variant of the *R*-selective IRED from *Myxococcus stipitatus* (R-IRED-*Ms*) was generated with a 2900-fold increase in NADH/NADPH specificity without the loss of activity.[98] Nevertheless, most applications of IREDs in the literature employ either NADPH regeneration systems or whole-cell transformations.

3.2.2.3 Stereoselectivity

In this section, the influence of substrate structure on the stereoselectivity of IRED-catalyzed reductions is discussed. Both the magnitude and the sense of the selectivity of a given IRED may vary depending on the substrate. Depending on the substituents of the imine carbon, the Cahn Ingold Prelog (*R*/*S*) assignment of absolute configuration can be different for products that were formed with visibly the same selectivity. This is why the *R* and *S* assignment of IREDs based on 2-methyl-1-pyrroline **28** cannot be used as such. However, the geometry of a cyclic substrate can usually be compared to 2-methyl-1-pyrroline **28** to rationalize the stereochemical outcome. These reactions are catalyst-controlled. For some IREDs, however, the stereochemical outcome is not catalyst-controlled and no predictions or rationalizations are possible. Some of the known examples of the latter are discussed below.

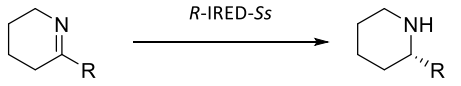
Small alterations of the substrate structure can have large impacts on stereoselectivity. This phenomenon is clearly illustrated by the reduction of dihydroisoquinolines **45** by *S*-IRED-*Ss* and *S*-IRED-*Sa*. A slightly larger substituent (R) on the imine carbon gives rise to a considerable drop in enantiomeric excess (*ee*) for both enzymes.[71]



45a R = Me — **46a** 98% *ee* (*S*-IRED-*Ss*); 99% *ee* (*S*-IRED-*Sa*)

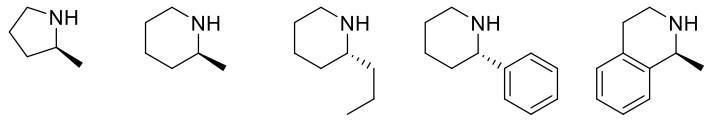
45b R = Et — **46b** 15% *ee* (*S*-IRED-*Ss*); 34% *ee* (*S*-IRED-*Sa*)

Another example of this is the reduction of 2-arylpiperideines **47** by *R*-IRED-*Ss*, where the selectivity is increased significantly by adding a substituent (*p*-Me, *p*-F, *p*-, *m*- and *o*-OMe) on the phenyl ring. The selectivity decreased when the methoxy substituent was moved from *para* to *meta* to *ortho*.[80]



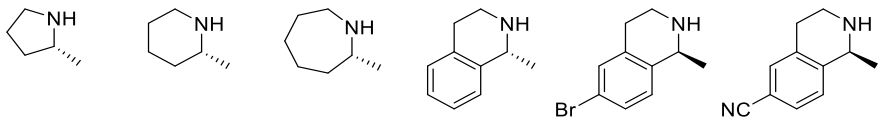
47a	R = Ph	**48a**	> 98%, 37% *ee* (*S*)
47b	R = *p*-Tol	**48b**	98%, > 98% *ee* (*S*)
47c	R = *p*-FC_6H_4	**48c**	98%, 91% *ee* (*S*)
47d	R = *p*-$MeOC_6H_4$	**48d**	> 98%, > 98% *ee* (*S*)
47e	R = *m*-$MeOC_6H_4$	**48e**	> 98%, 89% *ee* (*S*)
47f	R = *o*-$MeOC_6H_4$	**48f**	> 98%, 77% *ee* (*S*)

For some IREDs, the stereochemical outcome of a reaction is controlled by the substrate and can be inverted due to small changes in structure. The *S*-selective IRED from *Bacillus cereus* (*S*-IRED-*Bc*) catalyzes the formation of cyclic amines **29**, **48g**, **h** and **a** and **46a** with predictable stereoselectivity, with the hydride being delivered consistently to the same face of the ring (not shown). However, the *S*-selective IRED from *Nocardiopsis halophila* (*S*-IRED-*Nh*) displays an inversion in stereoselectivity for piperideine substrates with *n*-propyl and phenyl substituents on the imine carbon, resulting in amines (*R*)-**48h** and (*S*)-**48a**, respectively.[72]



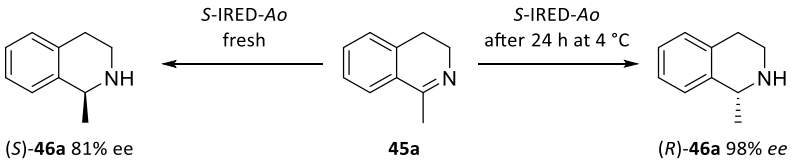
S-IRED-*Nh*	(*S*)-**29**	(*S*)-**48g**	(*R*)-**48h**	(*S*)-**48a**	(*S*)-**46a**
	> 99% *ee*	> 99% *ee*	82% *ee*	85% *ee*	68% *ee*

Substituents further away from the C=N bond can also influence the stereochemical outcome. The *R*-selective IRED from *Nocardia cyriacigeorgica* GUH-2 produces amines **29**, **48g**, **49a** and **46a** with predictable (*R*)-stereoselectivity. However, introduction of a 6-bromo or 6-cyano group on the 3,4-dihydroisoquinoline imine **45a** inverts the stereoselectivity and the (*S*)-enantiomers of 1,2,3,4-tetrahydroisoquinolines **46l** and **46m** are selectively formed. In the same study, *Streptomyces tsukubaensis* and *Streptomyces sp.* CNH287 (*R*)-IREDs behaved similarly and resulted in (*S*)-**46l**.[79]



IRED *Nocardia cyriacigeorgica* GUH-2	(*R*)-**29**	(*R*)-**48g**	(*R*)-**49a**	(*R*)-**46a**	(*S*)-**46l**	(*S*)-**46m**
	97% *ee*	98% *ee*	96% *ee*	87% *ee*	67% *ee*	35% *ee*

The *S*-selective IRED from *Amycolatopsis orientalis* gave equally unpredictable results with respect to small structural changes both in imine carbon substituent, as well as more remote alterations in cyclic imines. More importantly, the stereoselectivity of this enzyme toward some substrates changed depending on the storage time.



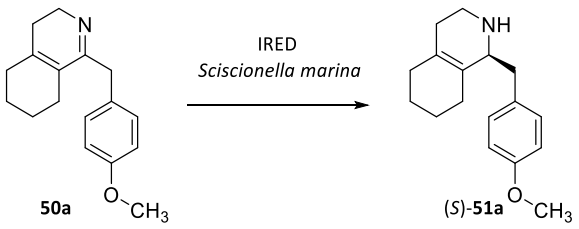
The reduction of 3,4-dihydroisoquinoline **45a** with freshly purified *S*-IRED-*Ao* resulted in (*S*)-**46a** with 81% *ee*, however, after 24 h of storage at 4 °C the stereoselectivity was inverted and (*R*)-**46a** was formed with 98% *ee*.[73]

3.2.2.4 Reaction conditions

Water-miscible cosolvents are often used in biocatalytic processes to increase the concentration of hydrophobic reagents in the aqueous reaction mixture.[101] With IREDs sometimes no cosolvent is used,[71,82] although usually they are. IREDs can typically tolerate up to 10% (*v*/*v*) of methanol, glycerol and dimethyl sulfoxide (DMSO), but are strongly inhibited by small amounts of ethanol, isopropanol, acetonitrile, acetone and *tert*-butanol.[65,84] Another cosolvent that is frequently used for IRED-catalyzed reactions is *N,N*-dimethylformamide (DMF).[67,72,80,86,102] The IRED from *Paenibacillus elgii* (*S*-IRED-*Pe*) was found to be very robust with respect to different cosolvents in high concentrations (up to 20% methanol).[84]

IREDs generally have optimal activity toward 2-methyl-1-pyrroline **28** at neutral pH. For *S*-IRED-*Pe*, *R*-IRED-*St* and *R*-IRED-*Sr,* the maximal activity was found at pH 7.0, and at pH below 5.5 and above 9.0 the activity was strongly reduced.[83,84] For *S*-IRED-*Ss* the optimal pH was found to be 7.0[65] and 7.5.[72] IREDs also catalyze the reverse reaction, namely the oxidation of amines to the corresponding imines. This reaction typically reaches maximal activity in more alkaline conditions with the optimal pH ranging from 8 to 11.[64,78,100]

The stereoselectivity of IRED-catalyzed reduction can be influenced by the pH as was seen for the reduction of imine **50a** with the IRED from *Sciscionella marina*. In this case the enantiomeric excess was highest at basic pH (98% *ee* at pH 9.0).[88]



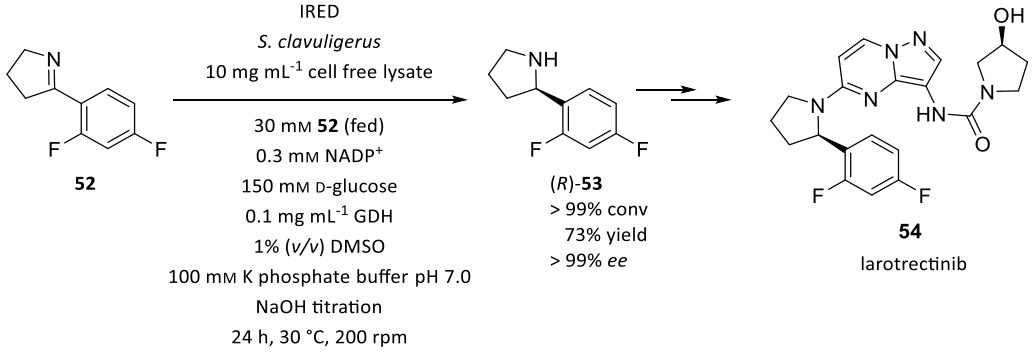
2.3.3 Imine reduction

3.2.3.1 Cyclic imines

IREDs are capable of reducing a wide range of cyclic imines. Unlike exocyclic and acyclic imines, these substrates are quite stable toward hydrolysis. When reaction with water occurs, the formed amino ketone readily recyclizes due to the proximity of the two reactive groups and the stabilizing effect of five- to seven-membered rings. It is for this reason that cyclic imines are the most studied substrates for IRED-catalyzed imine reductions.[103] An overview of the studied classes of cyclic imines is given.

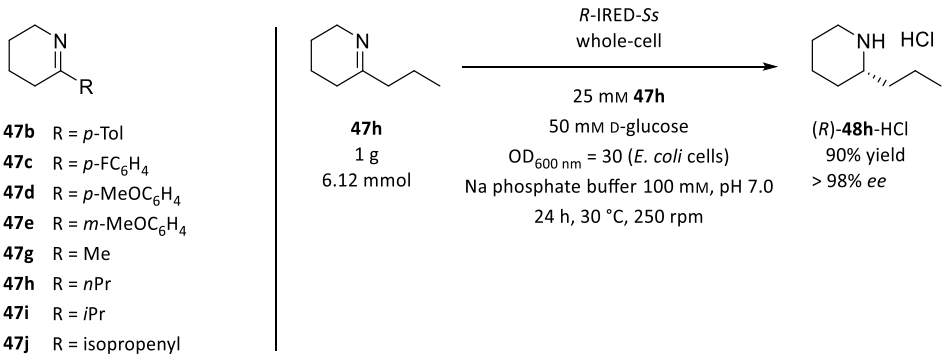
2-Substituted 1-pyrrolines

These five-membered cyclic imines were the first substrates to be successfully reduced by IREDs.[62,64,65] 2-Methyl-1-pyrroline **28** is used as a model substrate for IRED characterization, although IREDs typically have higher activities for six- and seven-membered imines.[67,68,72,81,84] A variety of 1-pyrrolines with larger substituents such as phenyl, *p*-methoxyphenyl, *p*-fluorophenyl, *p*-chlorophenyl, *o,m*-difluorophenyl and cyclohexyl (Cy) were successfully reduced to the corresponding pyrrolidines by IREDs.[73,80,86,87,104] Some *N*-substituted pyrroline iminium ions were reduced by *S*-IRED-*Ao* and *Asp*RedAm with low to moderate stereoselectivity.[73,74] Recently, (*R*)-2-(2,4-difluorophenyl)pyrrolidine (*R*)-**53**, a key building block for the cancer treatment drug larotrectinib **54** (LOXO-101),[105–107] was prepared in high yield and excellent selectivity using an IRED from *Streptomyces clavuligerus*.[104]



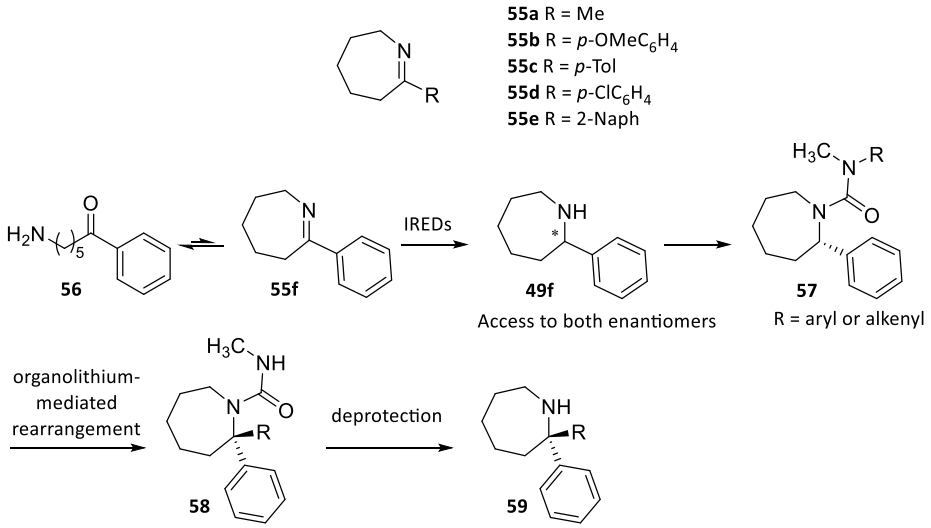
2-Substituted 1-piperideines

The reduction of 2-substituted 1-piperideines generates chiral piperidines. The latter are important scaffolds in many natural products.[108] Piperideines **47** with various substituents have been reduced with IREDs with excellent conversion and good to excellent stereoselectivity.[73,74,79,80,84,86,109] The alkaloid natural product (*R*)-coniine (*R*)-**48h** was synthesized at a preparative one gram scale using whole cells expressing *R*-IRED-*Ss*. A high yield and excellent selectivity was achieved.[80]

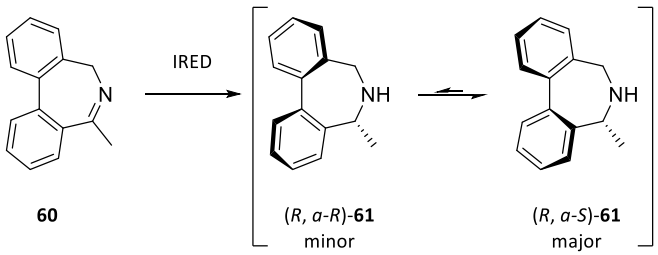


Seven-membered cyclic imines

Imines **55** have been successfully applied in IRED-catalyzed reductions with good conversion and excellent selectivities.[73,79,80,86,110] The aryl-substituted amine products were recently employed in the enantioselective synthesis of challenging α-tertiary amines **59**. The IREDs were used to create both enantiomers of azepane **49f**, which were converted into ureas **57** with various *N*-aryl groups. Ureas **57** underwent a stereospecific organolithium-mediated rearrangement involving a *N* to *C* aryl migration after benzylic lithiation and resulted after deprotection in 2,2-disubstituted azepanes **59**.[110]



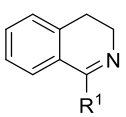
Dibenzo[*c,e*]azepines **60** can be reduced by IREDs to produce dihydrodibenzo[*c,e*]azepines **61** with excellent yield and selectivity.[76] These compounds have interesting conformational properties because of the axial chirality of the biaryl bond, which is imposed by substituents on the central ring.[111]



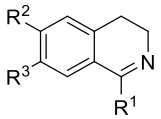
60 (*R*, *a*-*R*)-**61** minor (*R*, *a*-*S*)-**61** major

3,4-Dihydroisoquinolines

IREDs have been deployed to reduce 3,4-dihydroisoquinolines **45** to the corresponding tetrahydroisoquinolines. These compounds are important building blocks for some bio-active natural products and drugs.[112] Excellent conversions and stereoselectivities have been achieved for the reduction of 3,4-dihydroisoquinolines **45** with the depicted substitution patterns.[71,73,74,79,80,86,109,113] Through a screening, steric hindrance-tolerant IREDs were identified which reduce the challenging 1-aryl substrates with excellent conversion and *ee*.[113] The reduction of two *N*-methyl-3,4-dihydroisoquinoline iminium ions **62** have been attempted. The 1-methyl derivative **62b** was reduced with only low conversion and moderate selectivity by wild-type IREDs. However, the reaction was improved to 56% conversion and 99% *ee* (*R*) by mutation of *S*-IRED-*Ao*.[73,80,86]

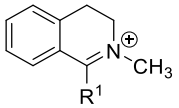


45a R^1 = Me
45c R^1 = H
45d R^1 = Ph
45e R^1 = *m*-Tol
45f R^1 = *m*-ClC_6H_4
45g R^1 = *m*-$OMeC_6H_4$



45k R^1 = Me, R^2 = R^3 = OMe
45l R^1 = Me, R^2 = Br, R^3 = H
45m R^1 = Me, R^2 = CN, R^3 = H

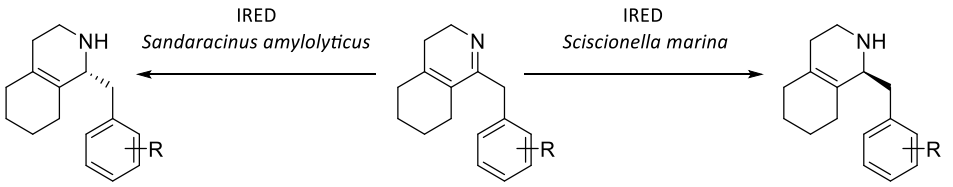
45h R^1 = *p*-Tol
45i R^1 = *p*-ClC_6H_4
45j R^1 = *p*-$OMeC_6H_4$



62a R^1 = H
62b R^1 = Me

3,4,5,6,7,8-Hexahydroisoquinolines

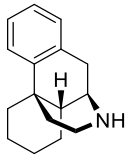
IREDs were recently used for the enantioselective synthesis of various 1-benzyl-1,2,3,4,5,6,7,8-octahydroisoquinoline derivatives **51** from the bulky α,β-unsaturated imines **50** (1-benzyl-3,4,5,6,7,8-hexahydroisoquinolines) at a preparative scale. These products **51** can be used for the synthesis of the pharmaceutically relevant morphinan scaffold **63**.[114,115] For example, (*S*)-**51a** and (*R*)-**51a** are intermediates for the synthesis of the APIs dextromethorphan and levallorphan, respectively. Two enantiocomplementary IREDs from *Sandaracinus amylolyticus* and *Sciscionella marina* were identified which can reduce these sterically demanding imines with high yields and excellent stereoselectivities. The IRED from *Sandaracinus amylolyticus* was found to be a particularly good enzyme for this transformation.[88]



(*R*)-**51a** 80% yield, > 99% *ee*
(*R*)-**51b** 82% yield, 99% *ee*
(*R*)-**51c** 95% yield, 99% *ee*
(*R*)-**51d** 90% yield, 99% *ee*

50a R = *p*-OMe
50b R = *p*-Cl
50c R = *o*-OMe
50d R = *o*-Cl

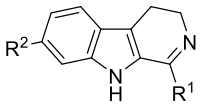
(*S*)-**51a** 74% yield, 98% *ee*
(*S*)-**51b** 70% yield, 94% *ee*



63

3,4-Dihydro-β-carbolines

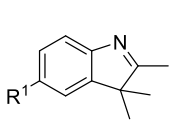
3,4-Dihydro-β-carbolines **64** are readily reduced by some IREDs to the corresponding (*S*)-tetrahydro-β-carbolines. These amines can be used as building blocks for biologically active substances.[116,117] Excellent conversions and selectivities were achieved with *S*-IRED-*Sa* and/or *S*-IRED-*Ss* for these imine substrates.[71,86]



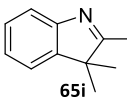
64a R^1 = Me, R^2 = H
64b R^1 = Et, R^2 = H
64c R^1 = *i*Pr, R^2 = H
64d R^1 = Cy, R^2 = H
64e R^1 = Me, R^2 = OMe

3*H*-indoles

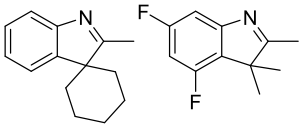
Two enantiocomplementary IREDs from *Paenibacillus lactis*, *S*-IRED-*Pl* and *R*-IRED-*Pl*, were identified for the reduction of 3*H*-indoles **65** and the corresponding *N*-alkyl iminium ions **66**. The resulting indolines are interesting compounds for medicinal chemistry.[112,118,119] A variety of these compounds were reduced with good to excellent conversion and excellent selectivities.[81]



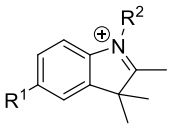
65a R^1 = H
65b R^1 = F
65c R^1 = Cl
65d R^1 = Br
65e R^1 = NO_2
65f R^1 = CF_3
65g R^1 = Me
65h R^1 = OMe



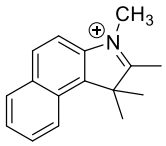
65i



65j **65k**



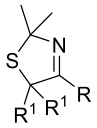
66a R^1 = H, R^2 = Me
66b R^1 = H, R^2 = Et
66c R^1 = H, R^2 = *n*Pr
66d R^1 = F, R^2 = Me
66e R^1 = R^2 = Me



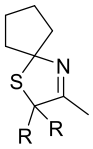
66f

Sulfur-nitrogen heterocycles

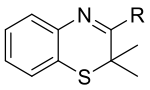
Recently, IREDs were used to produce synthetically challenging 3-thiazolidines and 2*H*-benzothiazines from 2,5-dihydrothiazoles **67** and 2*H*-benzothiazines **68**, respectively. These transformations occurred with good conversions (except for **68c**) and high selectivity.[85]



67a R = H, R^1 = Me
67b R = Me, R^1 = H
67c R = Me, R^1 = Me



67d R = H
67e R = Me

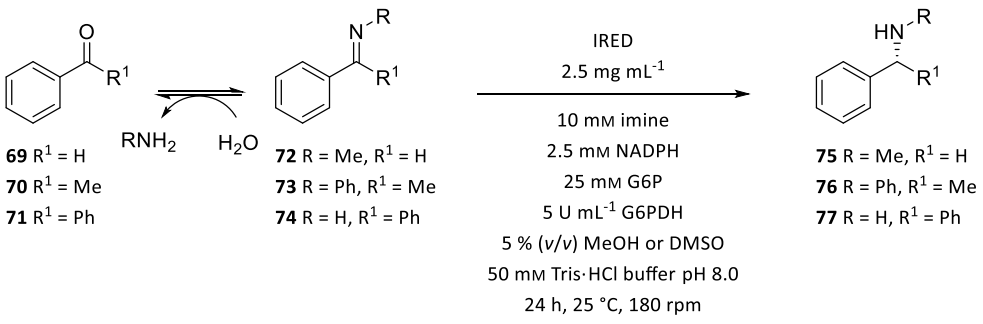


68a R = H
68b R = Me
68c R = Ph

3.2.3.2 Acyclic and exocyclic imines

The vast majority of the studied substrates for IRED-catalyzed reduction so far have been cyclic imines. However, in the scope of this thesis, acyclic imines are the most interesting substrates. Unfortunately, these substrates suffer from a poor stability in water, and thus there have been few reports on their enzymatic reduction. The general approach toward secondary and tertiary acyclic amines is reductive amination, where the imine intermediate is formed *in situ* and is subsequently reduced by the IRED, which will be discussed in '2.3.4 Reductive amination'. The few experiments of acyclic and exocyclic imine reduction that have been performed are summarized in this paragraph.

Aryl aldimine **72** was reduced by *R*-IRED-*Sr* with 76% conversion after 24 h, but it was not as well accepted by *R*-IRED-*St* and *S*-IRED-*Pe*. The prochiral ketimine **73** was best converted by *R*-IRED-*St*, however, *R*-IRED-*Sr* formed the product with a higher selectivity. Lastly, *S*-IRED-*Pe* was used to reduce achiral ketimine **74** with 53% conversion. Because of the low hydrolytic stability of these imines, a high catalyst concentration of 2.5 mg mL^{-1} had to be used.[83,84]



69 R^1 = H
70 R^1 = Me
71 R^1 = Ph

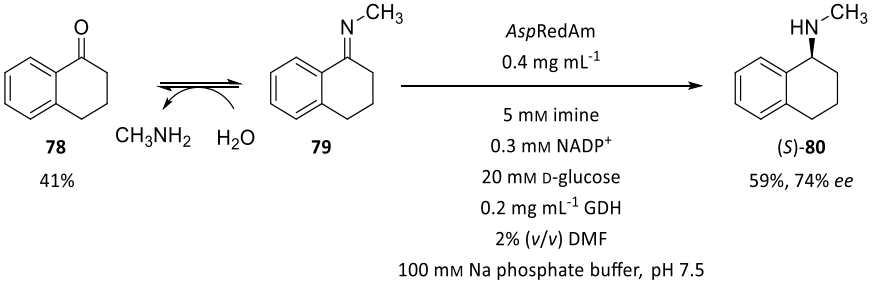
72 R = Me, R^1 = H
73 R = Ph, R^1 = Me
74 R = H, R^1 = Ph

75 R = Me, R^1 = H
76 R = Ph, R^1 = Me
77 R = H, R^1 = Ph

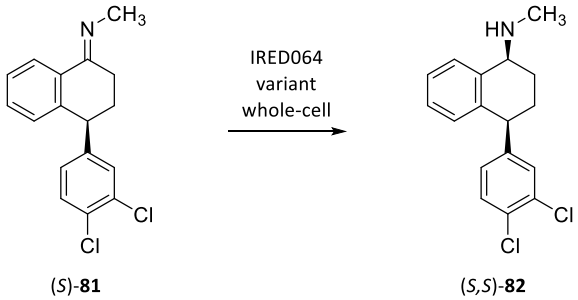
Enzyme	72	73		74
	conv (%) (hydr (%))	conv (%) (hydr (%))	*ee* (%)	conv (%) (hydr (%))
R-IRED-*Sr*	76 (10)[83]	10 (38)	94 (*R*)	53 (46)
R-IRED-*St*	57 (20)	84 (10)	88 (*R*)	-
S-IRED-*Pe*	14 (38)	-	-	-

Results from[84] and one from[83]
Conv = amine formation, Hydr = hydrolysis = carbonyl formation.
Carbonyl formations are likely underestimations due to basification during sampling.

The reductive aminase from *Aspergillus oryzae* (*Asp*RedAm) was used to reduce exocyclic imine **79**, however, only 59% was converted to amine (*S*)-**80** and 40% of imine **79** was hydrolyzed. The corresponding reductive amination, starting from ketone **78**, was also attempted, but resulted in only 5% conversion with methylamine added in 50-fold excess. This indicates that for this particular ketone/imine and enzyme combination, the enzymatic imine formation is the bottleneck.[74] However, this enzyme subclass (RedAms) could be interesting for the reduction of hydrolytically labile exocyclic imines, as they have been successfully applied in reductive aminations with equimolar amounts of carbonyl and amine (see section 2.3.4 Reductive amination).[74]



Pfizer developed an IRED variant originating from *Myxococcus fulvus* which is capable of forming the active pharmaceutical ingredient (API) (*S,S*)-sertraline (*S,S*)-**82** diastereoselectively from exocyclic imine (*S*)-**81**.[120]

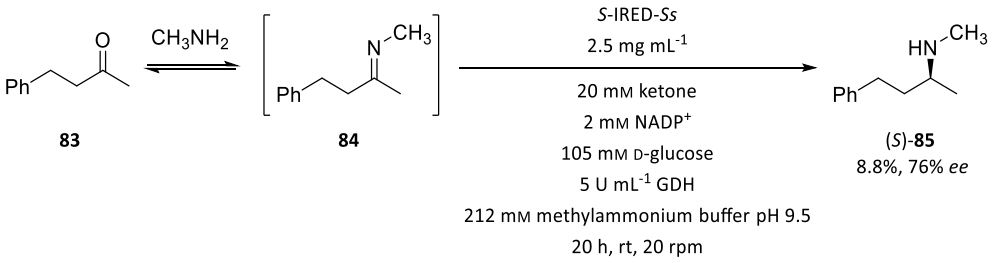


(*S*)-**81** (*S,S*)-**82**

2.3.4 Reductive amination

An alternative approach to produce (chiral) amines using IREDs is the reductive amination of ketones and aldehydes. In this method, the imine is generated *in situ* by condensation of a carbonyl compound and an amine, and is subsequently reduced to the corresponding amine product by action of the IRED.[58] This method is very attractive as it can generate exocyclic chiral secondary and tertiary amines from ketones in one step.

This was first attempted by Huber *et al.* by incubating a ketone, IRED, $NADP^+$, D-glucose and glucose dehydrogenase in methylammonium buffer. The best conversion (8.8%) was achieved with *S*-IRED-*Ss* and 4-phenylbutan-2-one **83** resulting in amine (*S*)-**85** in 76% *ee*. These reactions were highly pH dependent and the optimal pH was above 9 in all cases. The authors suggested that this basic pH could be necessary for the formation of imines in water. These results were not impressive, but did serve as a proof of concept for reductive aminations with IREDs.[71]



3.2.4.1 Imine formation

The first step in a reductive amination is the formation of an imine by condensation of an amine and a carbonyl compound. This reaction has been identified as the main bottleneck for enzymatic reductive amination with IREDs.[82,83] Since this is an equilibrium reaction with water as a side product, this reaction is highly disfavored when water is the solvent. In an aqueous environment this reaction proceeds best at alkaline pH, which ensures high concentrations of the amine in its non-protonated active form.[121,122] Consequently, reductive amination experiments are often performed at basic pH[71,82,83,97,123] despite the fact that IRED activity is typically maximal at neutral pH (see section 3.2.2.4 Reaction conditions). When imine formation is not catalyzed by the enzyme, imine formation and overall reaction rate have been observed to go up with nucleophilicity of the amine and with electrophilicity of the carbonyl compound.[83,97,99] Additionally, a large excess of amine is often used to push the equilibrium toward the imine intermediate.[71,82,83,91,124]

The formation of aldimine **72** by condensation of benzaldehyde **69** and methylamine in deuterium oxide at pD varying from 4.4 to 9.4 (pH 4 to 9) was studied using ^1H NMR spectroscopy (Figure 4).

At pD above 8.6 (pH > 8.2) the aldimine was formed substantially. However, when repeating the same experiment with acetophenone **70**, no ketimine could be detected, suggesting ketimine formation is less favorable than aldimine formation. Nevertheless, the reductive amination with *R*-IRED-*Sr* of acetophenone **70** and methylamine did proceed to some extent at pH 8 and 9.0. This means that this enzyme is efficient at withdrawing the imine intermediate at very low (< 500 μM) concentrations.[83] Similar observations were described for the reaction of hexan-2-one **92** with methylamine in water (pH > 9).[82]

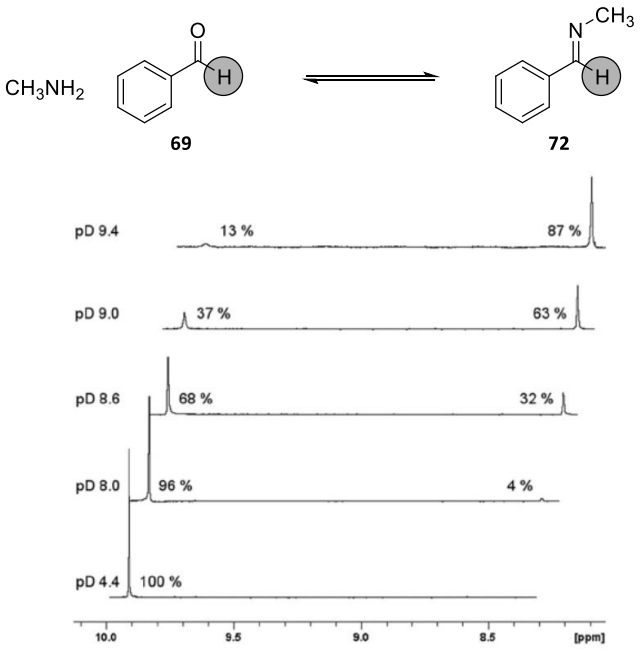


Figure 4. ^{1}H NMR (D_2O) measurement of equilibrium fractions of aldimine **72** and benzaldehyde **69** at different pD values.[83]

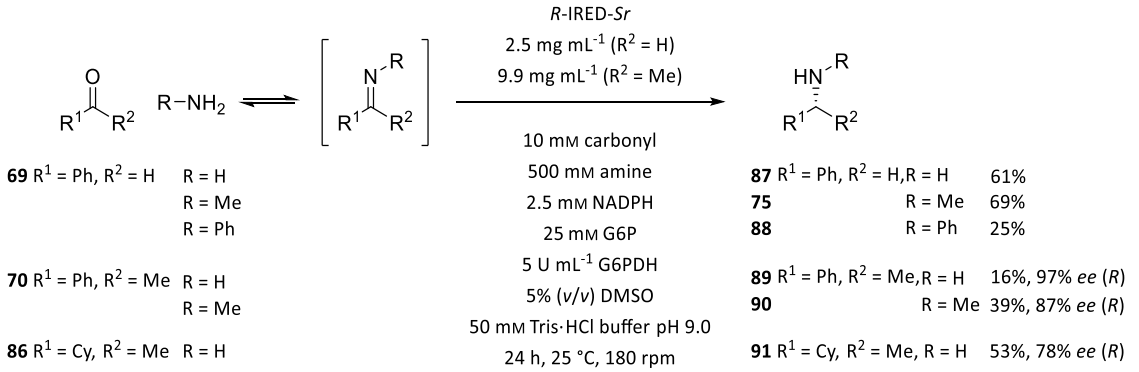
3.2.4.2 Reductive aminations using IREDs which are not RedAms

Primary amines

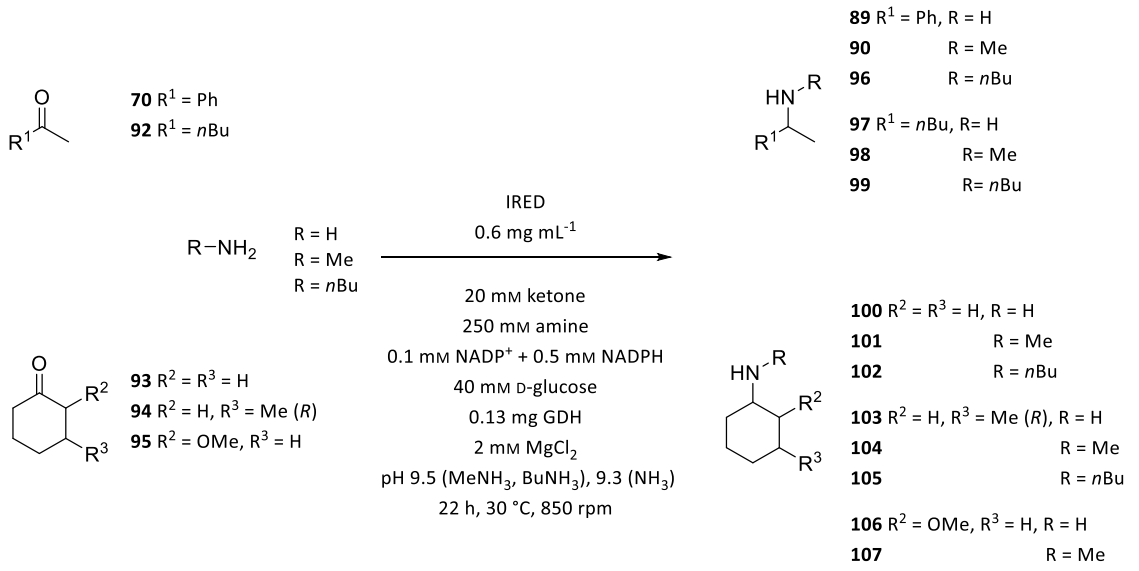
The reductive amination of benzaldehyde **69**, acetophenone **70** and cyclohexyl methyl ketone **86** was studied with the *R*-selective IRED from *Streptosporangium roseum R*-IRED-*Sr*. Benzaldehyde **69** was successfully converted to the amine products with all of the amine nucleophiles. Reaction rates and conversions were higher with more nucleophilic amines (aniline < ammonia < methylamine)[125] and with higher concentrations of amine. However, even with 50-fold excess amine the conversions did not exceed 70%.[83]

Reductive aminations of acetophenone **70** and cyclohexyl methyl ketone **86** proved more difficult. Even with 50 equivalents of amine and a fourfold increase in enzyme loading (0.78 mol% to 3.1 mol%), the conversions were low. The pH was most important for optimization of the reactions

with acetophenone **70**. The reactions with methylamine consistently had the best conversions and the stereoselectivity was highest for the reactions with acetophenone **70**.[83]



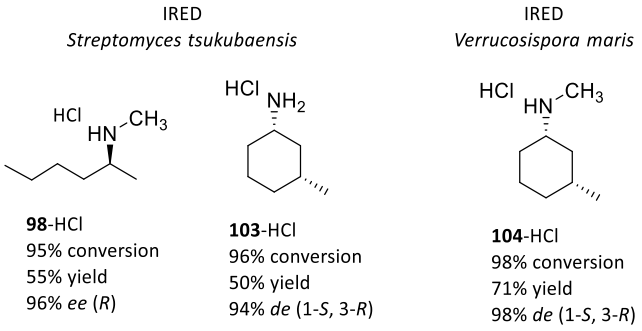
In a later study by researchers at Hoffmann-La Roche, 28 IREDs were screened for their reductive amination potential for five ketones **70**, **92**, **93**, **94** and **95**. The tested amine nucleophiles were ammonia, methylamine and butylamine. Due to the large number of screened IREDs, multiple ketone-amine combinations could be successfully transformed with a lower catalyst loading (0.6 mg mL^{-1}) and amine excess (12.5 eq) than in previous studies.[82]



With regard to the amine nucleophile, the reactions with methylamine were generally the most productive and those with butylamine were the least productive. As for the ketone substrate, acetophenone **70** led to poor conversions with almost all combinations of IREDs and amines. Hexan-2-one **92** was quite a good substrate and the cyclic ketones cyclohexanone **93**, (*R*)-2-methylcyclohexanone **94** and racemic 2-methoxycyclohexanone **95** were even better.

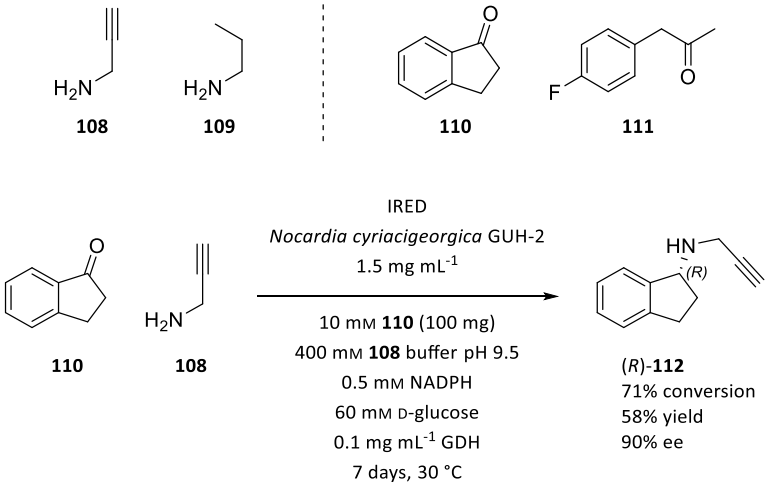
In the same study, hydrochloride salts of amines **98**, **103** and **104** were synthesized at a preparative (> 100 mg) scale using the IREDs from *Streptomyces tsukubaensis* and *Verrucosispora*

maris with excellent conversions and stereoselectivities and with moderate yields. The acidification from the gluconolactone and subsequent gluconic acid formation was countered by automatic titration with 1 M sodium hydroxide.[82]



Some of these experiments (only methylamine) were later repeated with lyophilized whole cells of *E. coli* expressing 13 previously described IREDs. This is an attractive alternative to the purified enzyme approach since it can cut production costs.[126] Only the ketones cyclohexanone **93** and 3-(*R*)-methylcyclohexanone **94** were converted with moderate to very high conversions and stereoselectivity.[123]

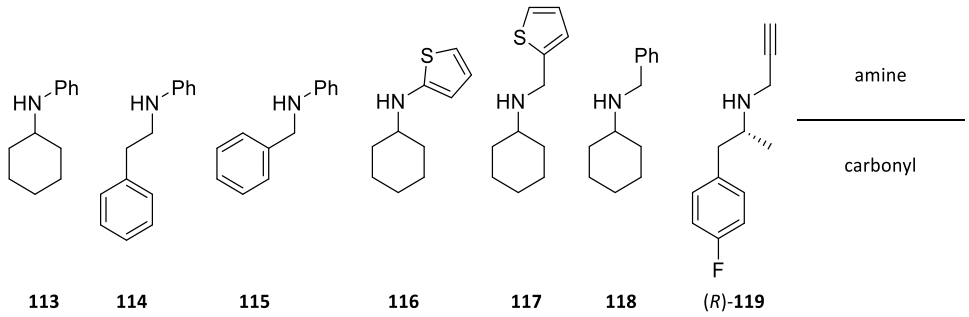
The substrate scope for reductive aminations with IREDs was further expanded in a series of publications. In a study by Matzel *et al.* propargylamine **108**, propylamine **109**, indanone **110**, and fluorinated ketone **111** were identified as good substrates for multiple IREDs and ketone or amine partners. The API (*R*)-rasagiline **112** was prepared on a 100 mg scale with moderate conversion and yield and good selectivity with the IRED from *Nocardia cyriacigeorgica* GUH-2.[124]



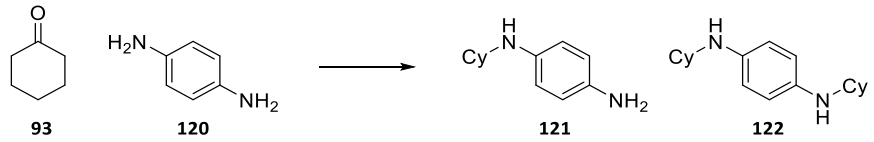
The same researchers later performed a more extensive photometric combinatorial screening for the IREDs from *Streptomyces tsukubaensis* and *Streptomyces ipomoeae* with 663 amine-carbonyl combinations. Cyclohexanone derivatives were clearly preferred over four-, five-, seven- and

eight-membered cyclic ketones, and keto acids proved to be bad substrates. Methylamine and ethylamine were the best amines for these two IREDs.[99]

The first (non-RedAm) IRED-catalyzed reductive aminations with equimolar amounts of amine (1.1 eq) by screening 85 enzymes were reported by researchers at GlaxoSmithKline. Aniline derivatives **113**, **114** and **115** were formed in excellent conversion by multiple IREDs. Thiophen-2-amine derivate **116** and benzylamine derivative **118** were formed with moderate conversion while for thiophen-2-ylmethanamine derivate **117**, high to excellent conversion was achieved. The (*R*)-enantiomer of amphetamine derivative **119** was obtained with moderate conversion and excellent enantioselectivity. Amine **119** could be methylated to afford a *para*-fluorinated derivative of API (*R*)-selegiline **12**.[97]

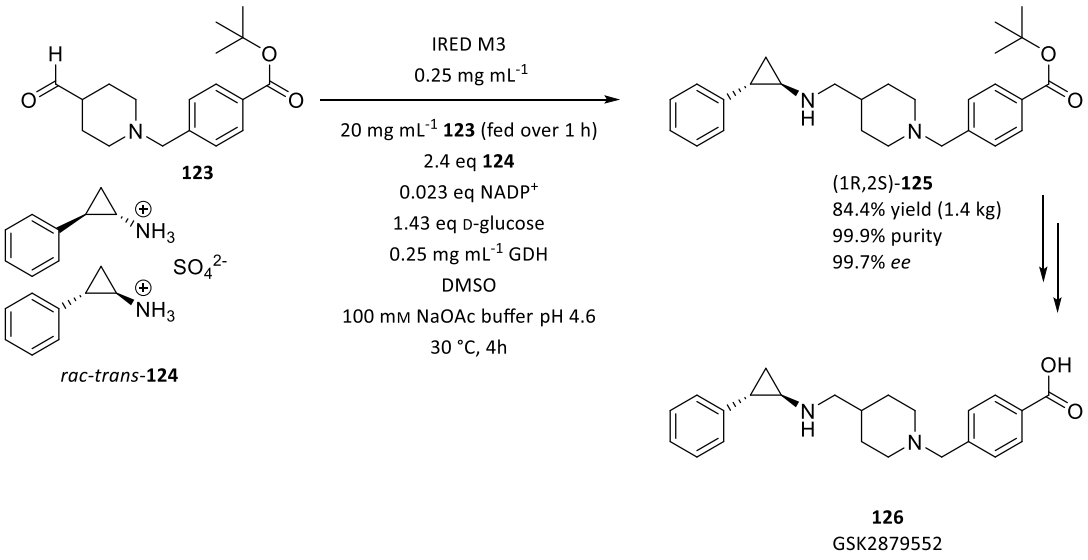


Reductive amination with symmetric aromatic diamine **120** resulted in regioselective formation of both the mono- **121** (IR-01, IR-10, IR-22, IR-49) and disubstituted product **122** (IR-13, IR-24). When two equivalents of cyclohexanone **93** were added, IR-01 and IR-10 retained high selectivity for the monosubstituted product **121** (99% and 97%, respectively).[97]



Recently an IRED was developed for the production of (1*R*,2*S*)-**125**, an intermediate in the synthesis of GSK2879552 **126**, a lysine-specific demethylase-1 (LSD1) inhibitor with potential applications in the treatment of acute leukemia and small-cell lung cancer.[127] The IRED was engineered to catalyze the reductive amination of aldehyde **123** with the racemic *trans*-cyclopropylamine **124** in a kinetic resolution where the (1*R*,2*S*)-aldimine is selectively reduced. The IRED from *Saccharothrix espanaensis* was improved 38 000-fold (k_{cat}) through three rounds of directed evolution (13 mutations) in order to be active under specific targeted operating conditions (acidic pH < 5, conversion > 95%, *ee* > 99.7%, aldehyde **123** loading 20 g L^{-1}). The final mutant (M3) was used in a large scale production with 1.4 kg of (1*R*,2*S*)-product isolated after

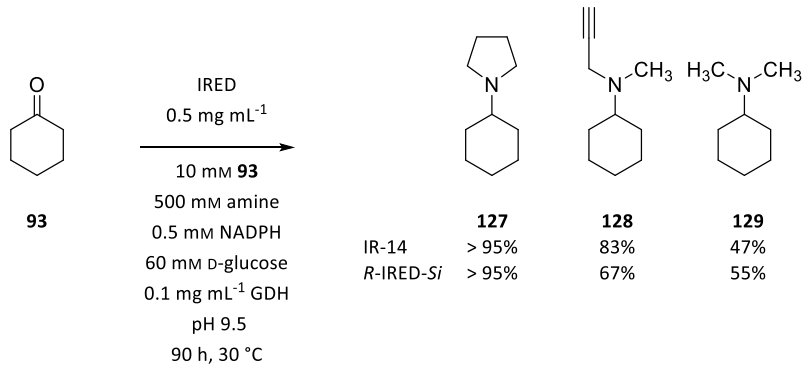
three 20 L batches with 84.4% yield, 99.9% purity and 99.7% *ee*. Interestingly, the racemic amine was added in only 2.4 equivalents (1.2 eq of the desired enantiomer) and the pH was 4.6, suggesting that imine formation might be catalyzed by this enzyme as well. This process is a clear improvement on the original chemical process, which employs a classical resolution of racemic amine **124** with (*R*)-mandelic acid followed by a reductive amination with $NaBH_4$.[92]



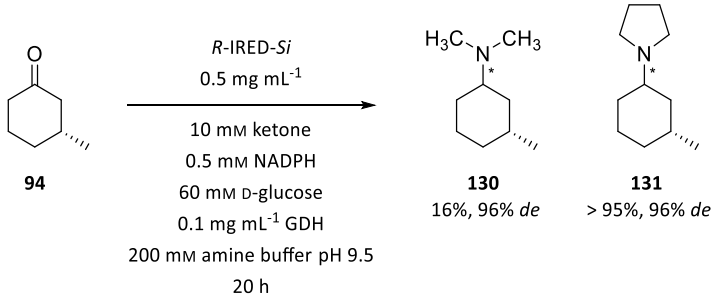
For more examples with low amine excess, see the literature.[91]

Secondary amines

Tertiary exocyclic amines are also accessible *via* reductive amination of carbonyl compounds using secondary amines. This was first accomplished with cyclohexanone **93** and *N*-methyl propargylamine, pyrrolidine and dimethylamine resulting in tertiary amines **127**, **128** and **129**, respectively, with the IREDs from *Nocardia cyriacigeorgica* GUH-2 (IR-14) and *Streptomyces ipomoeae* (*R*-IRED-*Si*). Pyrrolidine was shown to be a good substrate for this transformation.[124] Pyrrolidine is reported to be a particularly good amine for reductive aminations, and azepane has also been accepted to a lesser extent.[99,124]

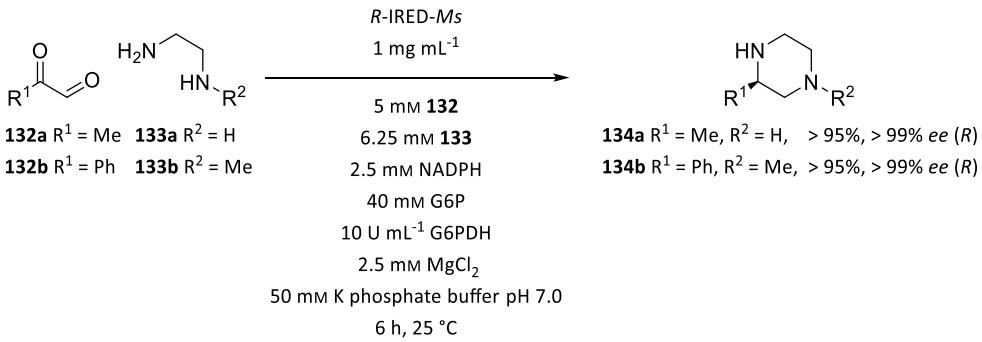


Attempts with (*R*)-3-methylcyclohexanone **94** with dimethylamine proved that tertiary amines (*e.g.* **130** and **131**) can be formed with high stereoselectivity using IREDs (absolute configuration not determined).[99]



Later, the reductive amination with secondary amines was also performed with equimolar (1.1 - 2 eq) amine addition. The tertiary amine **128** was formed by multiple IREDs but with low conversion (> 20%).[97]

Recently, a set of piperazines were synthesized *via* a double reductive amination with 1,2-diamines and 1,2-dicarbonyls using the *R*-selective IRED from *Myxococcus stipitatus*. For the diamines, *N*-alkyl substitution (*i.e.* secondary amines) was beneficial and *C*-substitution was detrimental. *C*-substituted dicarbonyls (*i.e.* ketoaldehydes and diketones) were converted better than oxalaldehyde. However, most diketones resulted in a mixture of diastereomeric piperazines due to racemization *via* imine-enamine tautomerization after the first reduction. The products **134a** and **134b** are potential building blocks for the APIs vicriviroc and mirtazapine, respectively, and were synthesized in high conversion and excellent regio- (**134b**) and stereoselectivity using this method.[128]



3.2.4.3 Reductive amination using reductive aminases (RedAms)

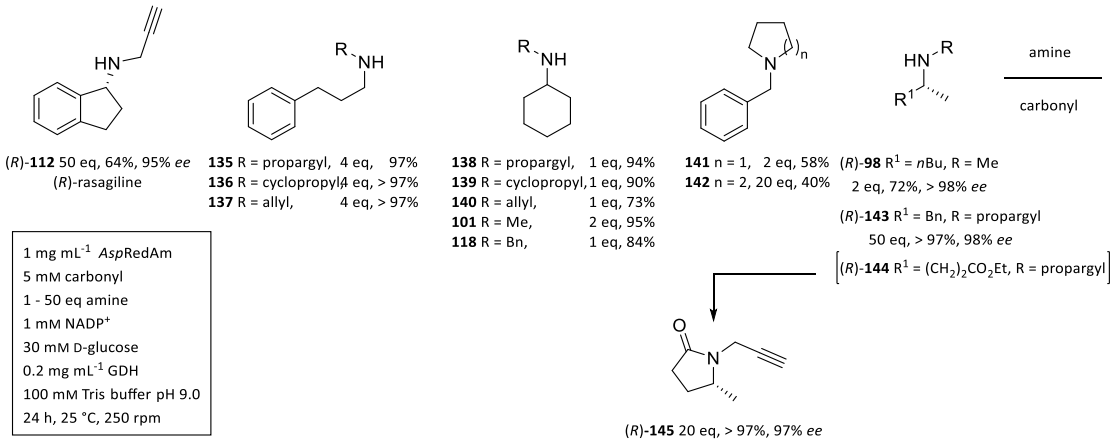
An important development in the field of enzymatic reductive amination is the discovery of a new subclass of IREDs of fungal origin called reductive aminases (RedAms). These enzymes were found to be capable of catalyzing reductive aminations without excess amine nucleophile present and at neutral pH. Thus they were hypothesized to catalyze the condensation of a carbonyl and an

amine in their active site, followed by the NADPH-dependent reduction of the formed iminium species.[89] This hypothesis was confirmed by elucidation of their kinetic mechanism (see section 2.3.1.2 Reductive aminases).[74]

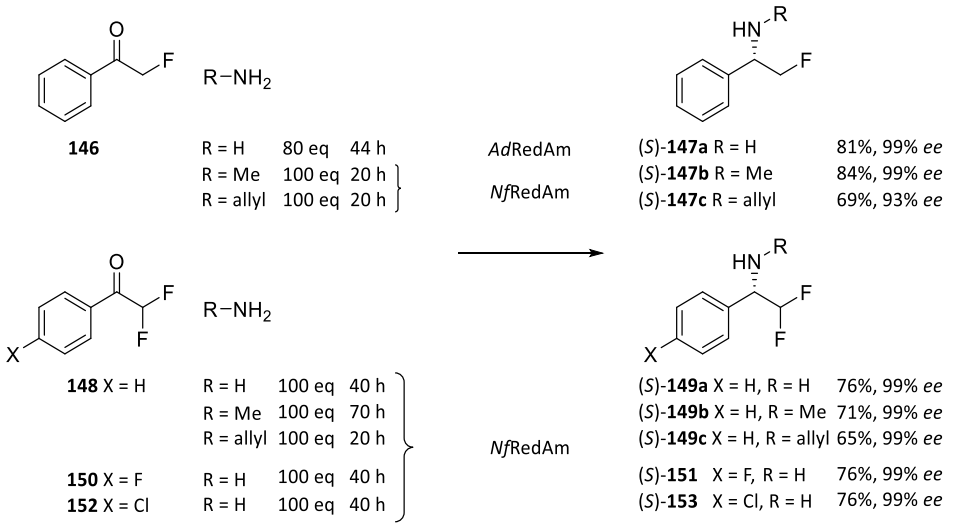
The enzymatic formation of the imine in the active site offers an advantage in that excess amine and high pH to raise the concentration of the imine intermediate in solution are not necessary. As long as the RedAm can bind with a suitable carbonyl compound and amine, the reaction will proceed. However, not all ketones and amines are readily accepted by a given RedAm. In that case the RedAm may operate at least partially in IRED-mode, *i.e.* reduction of a an imine intermediate formed in solution.[90]

The substrate scope of *Asp*RedAm was investigated by measuring its specific activity with a broad panel of amines and carbonyls. The carbonyls were all tested with two representative amines (propargylamine and methylamine) and the amines were all tested with two representative ketones (cyclohexanone **93** and 4-phenylbutan-2-one **83**). Based on these results a reactivity chart was constructed which was used to predict successful carbonyl-amine combinations. The best amines were cyclopropylamine, propargylamine and allylamine. These were preferred over acyclic primary alkylamines (methyl-, ethyl-, propyl- and butylamine), which indicates that nucleophilicity is not the determining factor here.[125,129,130] Furthermore, primary amines were clearly preferred over secondary amines. As for the carbonyl substrates, cyclic ketones like cyclohexanone **93** were preferred. Linear aldehydes and ketones (not conjugated) were also generally good substrates with a preference for C_5 and C_6 substrates over C_4. 1-Phenylpropan-2-one, a possible starting reagent for the synthesis of amphetamine derivatives, was one of the worst ketones, which presents an interesting challenge for this thesis.

Using the aforementioned reactivity chart, a variety of successful ketone-amine combinations were identified and attempted as shown below. Many of these reactions reached impressive conversions with equimolar or low amounts of amine and excellent enantioselectivity (where applicable). Interestingly, *Asp*RedAm was successful in reductive aminations of benzaldehyde **69** with cyclic secondary amines, resulting in moderate conversions to tertiary amines **141** and **142** with as low as twofold excess of pyrrolidine for tertiary amine **141**. γ-Lactam (*R*)-**145** was enantioselectively formed with excellent conversion by spontaneous cyclization after reductive amination of ethyl-4-octopentanoate with 20 equivalents of propargylamine. The API (*R*)-rasagiline **112** was produced by *Asp*RedAm from 1-indanone **110** with 64% conversion and 95% *ee*. Furthermore, the amphetamine derivative (*R*)-**143**, which may serve as a precursor for the API (*R*)-selegiline **12**, was synthesized in excellent conversion and selectivity.[74]



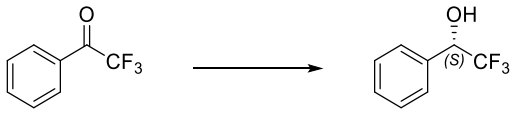
In a very recent study, the reductive amination of α-fluorinated (CH_2F, CHF_2, CF_3) aryl ketones **146**, **148**, **150**, **152** and **154** with ammonia, methylamine and allylamine using RedAms was investigated. Alcohol side products from promiscuous ketone reduction by RedAms was observed in most of these reactions. Aside from phenyl substituted ketones, *para*-halogenated substrates were also successfully converted. Among the tested enzymes the RedAms from *Ajellomyces dermatitidis* (*Ad*RedAm) and from *Neosartororya fumigatus* (*Nf*RedAm) were the best for these transformations. A large 80- to 100-fold excess was required for these transformations to reach high conversions. The stereoselectivity of these reactions was mostly excellent. For the *para*-halogenated substrates, the activity decreased with increasing halogen size, and no alcohol byproduct was produced.[90]



3.2.4.4 Promiscuous reduction of the carbonyl compound by IREDs

Carbonyl reduction activity from IREDs would be undesirable since it would produce alcohol side product, either from the hydrolyzed imine substrate in imine reductions or from the ketone substrate in reductive aminations. Reduction of carbonyl compounds to alcohols has been

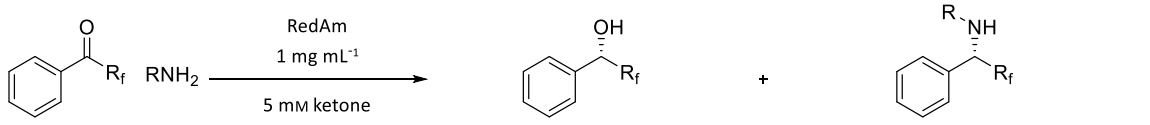
observed as a side reaction in several experiments with IREDs, but this was ascribed to the presence of ketone reductases owing to either incomplete protein purification[83,84,123] or the use of whole cells.[97,123] The activity of different IREDs toward aldehydes, ketones and ketoacids has been thoroughly investigated and in most cases no activity was detected.[64,78,80,131] However, the highly activated ketone **154** was substantially reduced by two IREDs.[132] This reaction is very relevant for this thesis, because trifluoromethyl ketones are expected to be formed from trifluoromethyl imines in an aqueous environment. Interestingly, the two IREDs had complementary stereoselectivity (*R*-IRED-*Sr* and *S*-IRED-*Pe*), yet both produced the (*S*)-enantiomer of alcohol **155** with high enantioselectivity.[131]



154 **155**

R-IRED-*Sr* 57%, 96% *ee* (*S*)
S-IRED-*Pe* 24%, 87% *ee* (*S*)

Recent investigations into the RedAm-catalyzed reductive amination of α-fluorinated acetophenone derivatives **146**, **148** and **154** (*cf. supra*) have revealed that the extent of ketone reduction versus reductive amination depends on the number of α-fluorine atoms (~ electrophilicity), the amine nucleophile, and the enzyme used. For acetophenone **70**, no alcohol formation was detected. For monofluorinated ketone **146**, alcohol **158** formation (3-19%) was only observed with ammonia and not with methylamine and allylamine. For difluorinated ketone **148**, alcohol **159** was the main product (81-98%) when ammonia was used, while for methylamine and allylamine the major product depended on the used RedAm. With trifluorinated ketone **154**, alcohol **155** was formed exclusively. *Para*-halogenated derivatives of these ketones were not converted to alcohols. All fluorinated alcohols were formed with high to excellent *S*-selectivity. For all of these ketones, RedAms are assumed to be operating in IRED mode, with the imine intermediates being formed in solution.[90]



70 $R_f = CH_3$
146 $R_f = CH_2F$
148 $R_f = CHF_2$
154 $R_f = CF_3$

(*R*)-**157** $R_f = CH_3$ (< 1%) (n.d.)
(*S*)-**158** $R_f = CH_2F$ (3-19%) (87-99% *ee*)
(*S*)-**159** $R_f = CHF_2$ (2-97%) (85-99% *ee*)
(*S*)-**155** $R_f = CF_3$ (1-> 99%) (90-99% *ee*)

(*R*)-**89**,**90**,**160** $R_f = CH_3$, (4-69%) (66-98% *ee*)
(*S*)-**147** $R_f = CH_2F$, (3-69%) (2-99% *ee*)
(*S*)-**149** $R_f = CHF_2$, (3-65%) (n.d)
(*S*)-**156** $R_f = CF_3$, (> 1%) (70-99% *ee*)

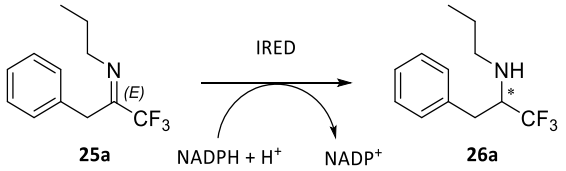
R = H, Me, allyl

2.4 Conclusion

IREDs and RedAms can be used to enantioselectively produce a variety of cyclic, acyclic and exocyclic chiral primary, secondary and tertiary amines either *via* imine reduction, or *via* reductive amination. α-Mono- and difluorinated amines have been successfully prepared in reductive aminations using multiple RedAms. However, attempts to obtain α-trifluorinated amines with these enzymes have been unsuccessful. This problem represents an important opportunity for scientific research in this field and provides an aim for this project.

3 Results and discussion

In this Master's thesis the enantioselective enzymatic reduction of trifluoromethyl imine **25a** was investigated using imine reductases (IREDs). In that respect, organic synthesis and biocatalysis were combined in an effort to produce trifluorinated amphetamine derivatives **26** in a sustainable way. The strong electron-withdrawing character of the trifluoromethyl group has a pronounced effect on the physicochemical and biological properties of molecules. For instance, in α-trifluoromethyl amines like amphetamine derivatives **26**, the amine nitrogen is significantly less basic than in non-fluorinated analogs.[42–44] This can result in interesting biological properties like enhanced blood-brain barrier penetration.[44–46]

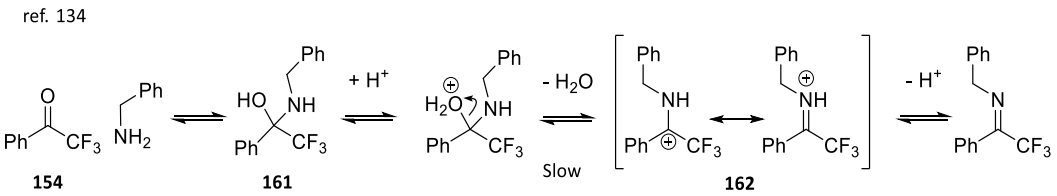


3.1 Synthesis of trifluoromethyl imines

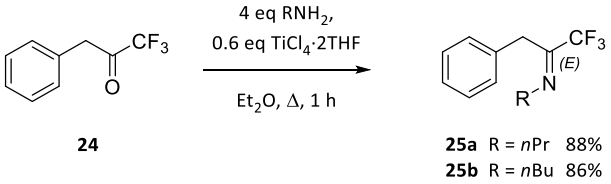
Trifluoromethyl imines are valuable building blocks for the synthesis of fluorinated nitrogen-containing biologically active compounds.[132,133] Due to the inductive electron-withdrawing effect of the CF_3 group, these imines are typically a lot more reactive than their non-fluorinated counterparts. Nucleophiles can therefore readily react with these imines without the need for *N*-activating substituents or Lewis or Brønsted acid activators.[132]

3.1.1 Synthesis

The condensation of primary amines with trifluoromethyl ketones has been reported to be difficult. It was suggested that the reaction involves a highly stabilized hemiaminal intermediate **161**, the dehydration of which results in a thermodynamically unstable intermediate **162**. This carbocationic/iminium species **162** is destabilized by the strong electron-withdrawing effect of the trifluoromethyl group. To speed up the reaction, activation techniques like high temperatures and acid catalysts have been used.[134,135]



In this Master's thesis, a synthesis method with titanium(IV) chloride as a Lewis acid activator and powerful dehydrating agent was slightly modified to synthesize trifluoromethyl imines **25**.[136,137] In that respect, imination of 1,1,1-trifluoro-3-phenylpropan-2-one **24** was performed in dry diethyl ether using four equivalents of primary amine (propyl- or butylamine) and 0.6 equivalents of the titanium activator. The solid tetrahydrofuran complex $TiCl_4$·2THF was used instead of liquid $TiCl_4$ because it is safer and easier to handle. The reaction was monitored with ^{1}H NMR analysis ($CDCl_3$) by looking at the benzylic proton signals, which are upfield in imines **25** compared to ketone **24**, and also with ^{19}F NMR ($CDCl_3$). Both imines were formed as single *E* isomers (*cf. infra*) and were isolated as orange oils without further purification in high purity and yield. In case of imine **25a** a less pure batch was obtained and purification by automated flash chromatography (SiO_2) was attempted. However, the imine was degraded. Thin layer chromatography (TLC) was also not possible for these imines as they both were unstable on the acidic stationary phase (SiO_2). Furthermore, attempts of altering the reaction conditions were also investigated, like different solvents (toluene, dichloromethane) and the use of an activator (none, *p*-toluenesulfonic acid). No hemiaminal intermediate mentioned in the literature[134,135] was observed with ^{1}H NMR spectroscopy in any of the attempted reaction conditions.



24

25a R = *n*Pr 88%
25b R = *n*Bu 86%

Table 1. Synthesis of (*E*)-trifluoromethyl ketimines **25**.

Compound	R	Conditions	Yield (%)
25a	*n*Pr	Dry Et_2O, Δ, 1 h	88
25b	*n*Bu	Dry Et_2O, Δ, 1 h	86

3.1.2 *E*/Z-Stereochemistry of trifluoromethyl imines

The stereochemical configuration of imines **25** was determined to be *E.* Some difficulties were encountered while doing this. Since the imines **25** were both liquids, X-ray analysis could not be used to determine the configuration. Also, only one isomer was formed, so no comparison could be made based on spectral data of both isomers (*e.g.* NOE size difference). One conceivable method to determine the configuration would be to compare the Nuclear Overhauser Effect (NOE) interaction between the NCH_2 protons and the two imine carbon substituents. However, there are no protons on one side (CF_3), so a ^{19}F-^{1}H heteronuclear NOE (^{19}F-^{1}H HOESY) experiment would be required, but this technology was not available in the lab.[138] In the end, it was decided

to compare the NOESY (Nuclear Overhauser Effect Spectroscopy) spectrum ($CDCl_3$) of these imines with the simulated minimal energy conformations of both the *E* and *Z* isomers, in hopes of eliminating one. The minimal energy conformations were simulated using the molecular mechanics (MM2) tool from Chem3D Pro (Figure 5).[139] Two significant NOE interactions were found for the NCH_2 protons (H_α) with the benzylic protons (H_{Bn}) and with the *ortho* aromatic protons (H_o) (Table 2). The NOESY spectrum of imine **25a** is shown in Figure 6, the same cross peaks were observed for imine **25b**.

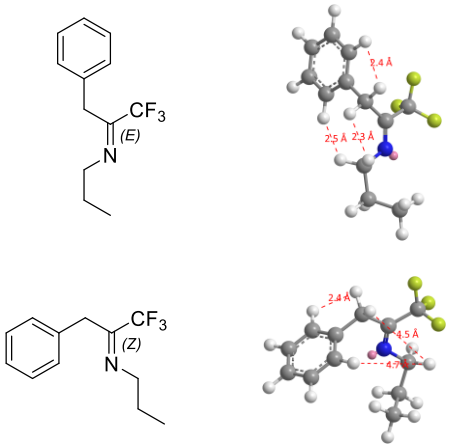


Figure 5. Simulated minimum energy conformations of the (*E*)- and (*Z*)-isomer of imine **25a**, calculated using molecular mechanics (MM2) in the Chem3D Pro 16.0 software (RMS value of 0.01, CambrigeSoft, PerkinElmer Informatics, Waltham, MA, USA).

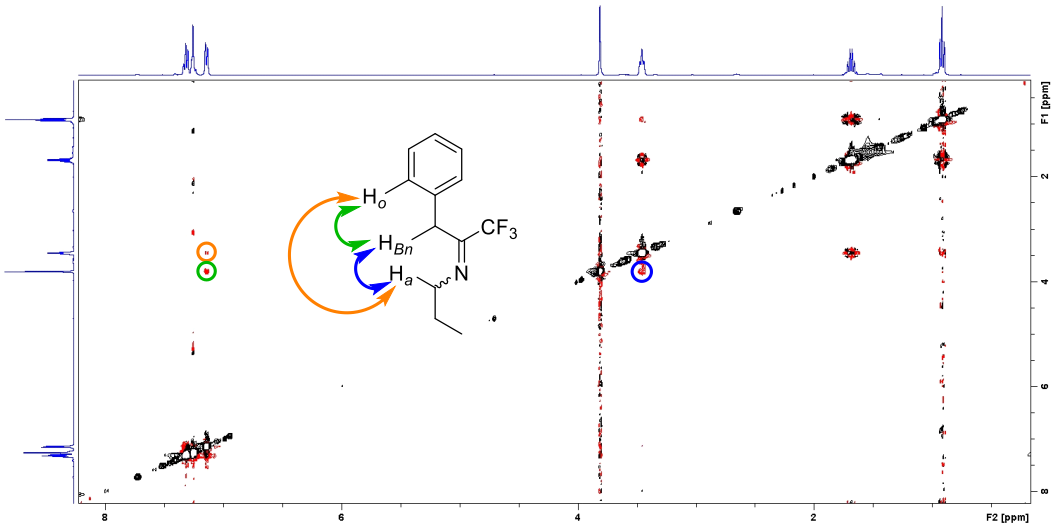


Figure 6. NOESY spectrum of imine **25a** ($CDCl_3$). The red cross peaks represent positive NOE coupling interactions.[138]

In both simulated isomers of imines **25a** and **25b**, the distances between the protons H_α - H_{Bn} and H_α - H_o are within the critical range for NOE (5 Å), so no isomer can be definitively ruled out. However, the distances for the *E* isomers are significantly smaller and thus more likely to exhibit NOE.

Table 2. Determination of the E/Z stereochemistry of trifluoromethyl imines **25**.

			Internuclear distance r (Å)[a]		
Imine	**R**	**Configuration**	**H_a - H_{Bn}**	**H_a - H_o**	**H_o - H_{Bn}**
25a	*n*Pr	*E*	2.3 ± 0.0	2.5 ± 0.0	2.4 ± 0.0
		Z	4.5 ± 0.0	4.7 ± 0.1	2.4 ± 0.0
25b	*n*Bu	*E*	2.2 ± 0.1	2.8 ± 0.1	2.4 ± 0.0
		Z	4.5 ± 0.0	4.8 ± 0.0	2.4 ± 0.0

[a] The protons with the smallest mutual distance where chosen (*e.g.* there are two H_a and two H_{Bn} protons). Simulations were performed in triplicate with different initial conformations. The average distances and standard deviations are given.

To further substantiate the (*E*)-stereochemistry assignment, a third interaction in the NOESY spectra was considered, namely the benzylic protons H_{Bn} and the *ortho* protons H_o. The H_{Bn} - H_o distance was consistently 2.4 ± 0.0 Å for all four simulated species. The size of the NOE between two nuclei is dependent on their distance in space (~ r^{-6}), among other factors.[138] Thus the integration values of the three cross peaks in the NOESY spectra of imines **25a** and **25b** were plotted as a function of their calculated distances^{-6} to select the isomer with the best fit (Figure 7). Visually, it is clear that the (*Z*)-conformation is not likely to be correct. The strongest measured NOE interaction for both **25a** and **25b** (H_a - H_{Bn}) would be at 4.5 Å, instead of the much closer H_o - H_{Bn} interaction at 2.4 Å. The simulated (*E*)-isomers gave a much better fit, where the NOE is stronger for protons which are closer together in space. Since the energy minimizations were performed with relatively simple molecular mechanics calculations instead of more sophisticated *ab initio* methods, the calculated distances of the energy minimized structures are unlikely to be exactly correct. However, considering the small size and simplicity of the molecules, it can be confidently concluded that imines **25a** and **25b** possess (*E*)-stereochemistry.

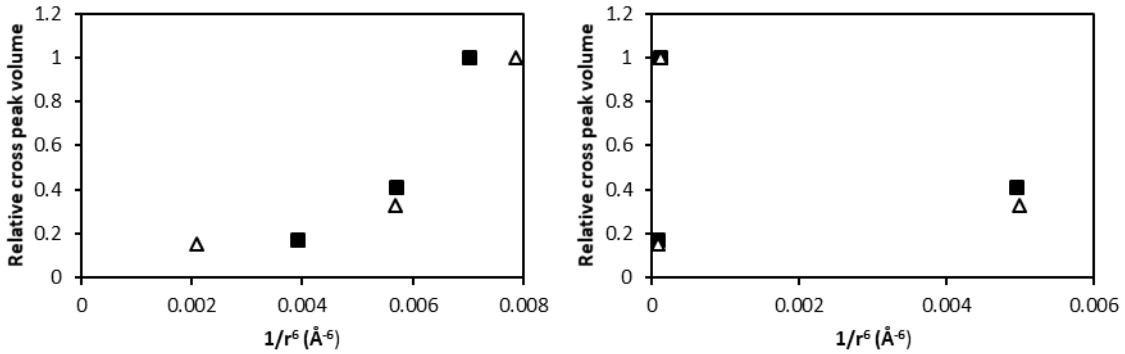


Figure 7. NOESY cross peak integration values in function of the corresponding calculated internuclear distances^{-6}. Left: the (*E*)-isomers. Right: the (*Z*)-isomers. ■ **25a** (*n*-propyl), △ **25b** (*n*-butyl).

3.2 Reduction of trifluoromethyl imines

Fluorinated amines represent an important class of compounds in medicinal chemistry. The presence of fluorine atoms in amines can drastically alter their physical, chemical and biological

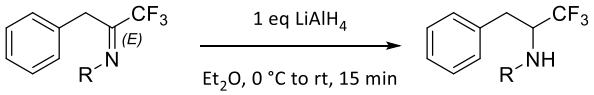
properties. Due to its ultimate electronegative character,[41] fluorine inductively withdraws electron density, resulting in amines with lowered basicity.[42–44] These amines are therefore more likely to be present in neutral form at physiological pH, which can change *in vivo* interactions, improve oral bioavailability and enhance uptake through the blood-brain barrier.[44–46] β-Fluorinated amines have been shown to be potent inhibitors of pyridoxal 5'-phosphate (PLP)-dependent enzymes.[36] Furthermore, α-trifluoromethyl amines have also been used as metabolically stable isosteres for amide and peptide bonds due to the isopolarity of C-CF_3 and C=O functionalities.[37–40]

Multiple chemical enantioselective synthesis methods for these trifluoromethyl amines have been reported. One possibility is the enantioselective reduction of trifluoromethyl ketimines or enamides with transition metal catalysts using high pressure hydrogen gas[133,140,141] or organic hydrogen donors[142,143] (transfer hydrogenation). Enantioselective hydride additions to trifluoromethyl ketimines using a chiral catalyst[135] or a chiral auxiliary[144] have also been reported. Another route is the addition of organometal nucleophiles like Grignard,[145–147] organolithium,[147,148] organozinc[149] and boronic acid reagents[150–152] to trifluoromethyl imines or their *N*,*O*-acetals. Yet another method is the cinchona alkaloid derivative-catalyzed enantioselective isomerization of ketimines to chiral aldimines *via* a proton shift, followed by hydrolysis to release the corresponding primary amines.[153–155] This sparked the development of several umpolung approaches, which involve the enantioselective addition of carbon electrophiles to 2-azaallyl anions generated from trifluoromethyl imines.[156–160] Lastly, there is the asymmetric nucleophilic trifluoromethylation of imines with trimethyl(trifluoromethyl)silane.[161–163]

There has been less development in terms of enzymatic approaches towards chiral trifluoromethyl amines. The (dynamic)[164–166] kinetic resolutions of trifluoromethyl amines to the corresponding enantioenriched *N*-acyl derivatives using lipases have been reported.[164–167] During this project, the reductive amination of α-fluorinated acetophenone derivatives using RedAms was reported (see section 3.2.4.3 Reductive amination using reductive aminases (RedAms)). With trifluoromethyl ketones, however, exclusively alcohol product (< 1% imine reduction) was formed by promiscuous IRED-catalyzed ketone reduction.[90] In conclusion, there have been no successful enzymatic reductions of trifluoromethyl imines reported in the literature, which is why this project could potentially provide some valuable new insights.

3.2.1 Chemical, non-stereoselective reduction of trifluoromethyl imines

Imines **25** were chemically reduced to produce amines **26**. These racemic products were needed as reference materials to determine the product formation and enantiomeric excess of later enzymatic reactions as well as for full product characterization. Attempts with sodium borohydride in isopropanol were unsuccessful, with no product formation after 24 hours. Next, the reaction was attempted with lithium aluminum hydride, a stronger reducing agent. This was successful and complete conversion (^{1}H and ^{19}F NMR analysis, $CDCl_3$) was achieved after 15 minutes.



25a R = *n*Pr
25b R = *n*Bu

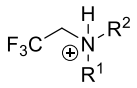
26a R = *n*Pr 95%
26b R = *n*Bu 72%

Table 3. Synthesis of trifluoromethyl amines **26**.

Compound	R	Conditions	Yield (%)[a]
26a	*n*Pr	Dry Et_2O, 0 °C to rt, 15 min	95
26b	*n*Bu	Dry Et_2O, 0 °C to rt, 15 min	72

[a] Crude yields.

After workup, the purity of the secondary amines **26a** and **26b** was already quite high (> 90% ^{1}H NMR analysis, $CDCl_3$). However, a higher purity is required for usage as internal standard or as product standard for calibration curves. Therefore it was attempted to purify these amines by automated flash chromatography. First, amine **26a** was subjected to reversed phase flash chromatography (C18), showing good separation. After rotary evaporation to remove the solvent (a mixture of acetonitrile and water) abysmal yields were obtained, with no detectable product (^{1}H NMR analysis, $CDCl_3$). The amine **26a** was more volatile than initially expected from its molecular weight and amino group, as it co-evaporated with the water. It was reasoned that due to the electron-withdrawing nature of the trifluoromethyl group, the electron pair on the nitrogen was made less available for hydrogen bonding. This phenomenon is somewhat comparable with the effect of β-fluorine substitution on the basicity of amines.[44] For example, the trifluoromethyl amines **163**, **164** and **165** are significantly less basic than non-fluorinated amines ($pK_a \sim 10$).[42,43]



163 $R^1 = R^2 = H$	pKa = 5.7	ref. 43
164 $R^1 = Me$, $R^2 = H$	pKa = 6.05	ref. 42
165 $R^1 = R^2 = Me$	pKa = 4.75	ref. 42

To avoid losing the product, a normal phase automated flash chromatography (SiO_2) was attempted, which employs apolar and volatile solvents. A custom gradient program with petroleum ether to ethyl acetate resulted in product that was contaminated with grease from the petroleum ether. Therefore, in a later attempt to purify amine **26b**, the petroleum ether was replaced with hexane, and a good yield (99%) and high purity (> 97%) was achieved. Amine **26a** had to be purified using manual column chromatography (SiO_2), because the automated flash device was out of use for several weeks. This resulted in incomplete separation of the impurities from the product, although enough pure product was obtained for spectral characterization. In the end, due to lack of time (COVID-19 lockdown) the calibration curve of amine **26a** was made using crude product (> 93% purity due to grease).

As mentioned above, it is also possible to chemically reduce trifluoromethyl imines in an enantioselective manner.[133,135,140–143,168] However, in this project the focus will lie on the development of an enzymatic approach in hopes of avoiding the disadvantages associated with the chemical approach.

3.2.2 Enzymatic reduction of trifluoromethyl imines

The enzymatic reduction of imine **25a** was studied with four imine reductases (IREDs) originating from *Paenibacillus elgii* B69, *Streptomyces sp.* GF3546, *Streptomyces ipomoeae* 91-03 and *Streptomyces sp*. GF3587. These enzymes catalyze the reduction of imines through the addition of a hydride from the nicotinamide cofactor NADPH to the imine carbon. The recent development of this class of enzymes has brought about huge advances in the field of enzymatic imine reduction and reductive amination, two reactions which were long thought to be unfeasible due to the instability of imines in aqueous environments. However, cyclic imines like 2-methyl-1-pyrroline **28** are hydrolytically stable and have been successfully reduced with good conversion and enantioselectivity. For noncyclic imines like those studied in this thesis, the method of choice in the literature is *in situ* formation of the imine by condensation of a carbonyl compound and an amine followed by IRED-catalyzed reduction.[56,57] In this thesis, however, it was decided to first attempt the two-step approach of chemically synthesizing the imines and subsequently reducing them enzymatically.

Two *S*- and two *R*-selective IREDs were purchased from Enzymicals AG as enzyme solutions. In order to be able to compare the properties of these enzymes (*e.g.* specific activity), their protein concentration had to be determined by a photometric measurement using the Pierce BCA™ Protein Assay kit (Table 4).

Table 4. The imine reductases (IREDs) employed in this project.

Enzyme	Alias[a]	Protein concentration (mg mL^{-1})
Paenibacillus elgii B69	*S*-IRED-*Pe*	16
Streptomyces sp. GF3546	*S*-IRED-*Ss*	20
Streptomyces ipomoeae 91-03	*R*-IRED-*Si*	20
Streptomyces sp. GF3587	*R*-IRED-*Ss*	18

[a] The *R*/*S*-selectivity annotation is based on the enzyme's selectivity toward 2-methyl-1-pyrroline **28**.

*3.2.2.1 Activity of IREDs with 2-methyl-1-pyrroline **28***

The specific activity of the four IREDs toward the standard substrate **28** was determined photometrically by measuring the rate of NADPH consumption at 340 nm, *i.e.* measuring the decrease in absorbance. One unit of enzyme activity (U) was defined as the amount of protein that oxidizes 1 µmol of NADPH per minute, under the conditions described in Table 5. The influence of cosolvent was investigated and it was determined that DMF was superior to DMSO in this particular reaction for all four of the IREDs. The obtained specific activity values were also compared with literature results and apart from *R*-IRED-*Ss*, the specific activities were of the same order of magnitude. Variations can occur due to differences in reaction conditions like temperature and imine **28** concentration.

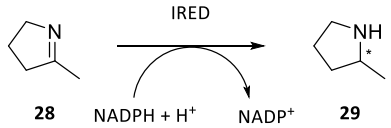


Table 5. Activities of IREDs with standard substrate 2-methyl-1-pyrroline **28**.

Enzyme	Activity DMSO (U mg^{-1})[a]	Activity DMF (U mg^{-1})[a]	Literature (U mg^{-1})
S-IRED-*Pe*	0.022 ± 0.001	0.029 ± 0.009	0.04[84]; 0.020[78]
S-IRED-*Ss*	0.015 ± 0.004	0.017 ± 0.002	0.13[65]; 0.050[78]
R-IRED-*Si*	0.018 ± 0.002	0.021 ± 0.009	0.027[78]
R-IRED-*Ss*	0.030 ± 0.001	0.032 ± 0.001	10.2[64]; 0.490[78]

[a] Activity assays were performed in triplicate, with 1.5 mg mL^{-1} IRED, 5 mM **28**, 750 µM NADPH, 50 mM sodium phosphate buffer, pH 7.0, 1% (*v*/*v*) cosolvent at 20 °C.

3.2.2.2 Activity of IREDs with trifluoromethyl imines

Next, the activity of the IREDs toward trifluoromethyl imine **25a** was determined in a similar way as the activity assay with 2-methyl-1-pyrroline **28**. The activity toward imine **25a** was significantly

lower for all four of the IREDs, although two of the enzymes, *R*-IRED-*Si* and *R*-IRED-*Ss,* were still quite active toward imine **25a** with a specific activity of about 0.012 U mg^{-1}. Relative to the reduction of 2-methyl-1-pyrroline **28**, *R*-IRED-*Si* and *R*-IRED-*Ss* retained 58% and 40% activity, respectively. Unfortunately, both of these IREDs are *R*-selective toward 2-methyl-1-pyrroline **28** and are thus expected to both have the same selectivity for the reduction of imine **25a**. *S*-IRED-*Pe* did retain some activity (9%), but *S*-IRED-*Ss* can be considered inactive based on the performed experiments. *N*,*N*-Dimethylformamide (DMF) was selected as the cosolvent for this reaction based on its frequent use in the literature.[67,72,80,86,102] However, in future research the reaction may be optimized by attempting different cosolvents (*e.g.* DMSO, toluene, dioxane, acetonitrile, methanol etc.) at different concentrations. In a recent study, acetonitrile and methanol were shown to be suitable cosolvents for the RedAm-catalyzed reductive amination of α-fluorinated ketones.[90] Therefore, these cosolvents may also be beneficial in the IRED-catalyzed reduction of trifluoromethyl imine **25a**.

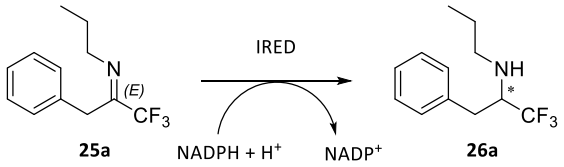


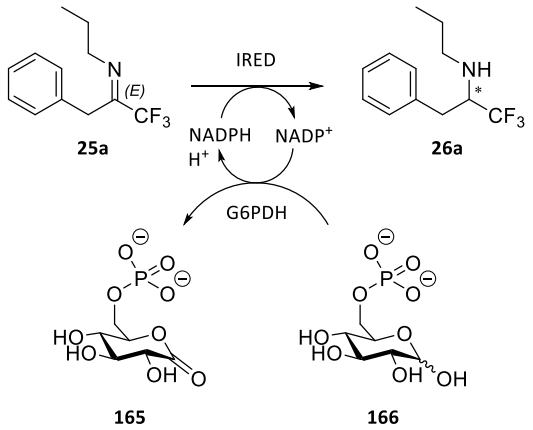
Table 6. Activity of IREDs with trifluoromethyl imine **25a**.

Enzyme	Activity (U mg^{-1})[a]	Relative activity (%)[b]
S-IRED-*Pe*	0.0027 ± 0.0011	9
S-IRED-*Ss*	0.0007 ± 0.0009	4
R-IRED-*Si*	0.0121 ± 0.0004	58
R-IRED-*Ss*	0.0119 ± 0.0002	40

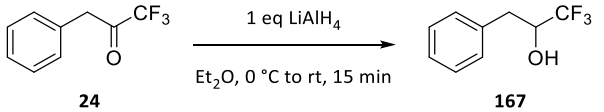
[a] Activity assays were performed in triplicate, with 1.5 mg mL^{-1} IRED, 5 mM **25a**, 750 μM NADPH, 50 mM sodium phosphate buffer, pH 7.0, 2% (*v*/*v*) DMF at 20 °C.
[b] This is the specific activity relative to the enzyme's specific activity with the standard substrate 2-methyl-1-pyrroline **28**.

3.2.2.3 Reduction of trifluoromethyl imines with cofactor regeneration

Having proved considerable activity of two IREDs for the reduction of trifluoromethyl imine **25a**, the logical progression is to attempt an experiment with cofactor regeneration. This way the reaction can theoretically reach full conversion or at least measurable product concentrations, without having to add stoichiometric amounts of expensive NADPH. To that end, glucose-6-phosphate dehydrogenase (G6PDH) and an excess of glucose-6-phosphate (G6P) **166** were added.



There are multiple processes beside imine reduction that can occur in this reaction setup, and some of these potential reactions are displayed in the scheme below.[99] Past studies of IRED-catalyzed exocyclic imine reductions invariably report substantial imine hydrolysis with formation of carbonyl and primary amine side products.[74,83,84] In the case of trifluoromethyl imine **25a**, the hydrolysis would result in a very reactive ketone **24**. There have been multiple reports of alcohol formation due to promiscuous ketone reduction activity of IREDs for activated α-fluorinated aromatic ketones (see section 3.2.4.4 Promiscuous reduction of the carbonyl).[90,131] Therefore, formation of alcohol **167** might be possible. To confirm or rule out its enzymatic formation, trifluoromethyl alcohol **167** was chemically synthesized *via* reduction of ketone **24** with lithium aluminum hydride.



It is also known that IREDs can oxidize amines in the presence of $NADP^+$ to form imines, which can subsequently be hydrolyzed to the corresponding amines and carbonyl compounds (oxidative deamination). It is important to note, however, that oxidation reactions are unlikely due to the presence of excess glucose-6-phosphate, which favors the reduction reactions. Moreover, IREDs are more efficient at reducing imines than at oxidizing amines, especially at neutral pH (see section 3.2.2.4 Reaction conditions).[64,78,100]

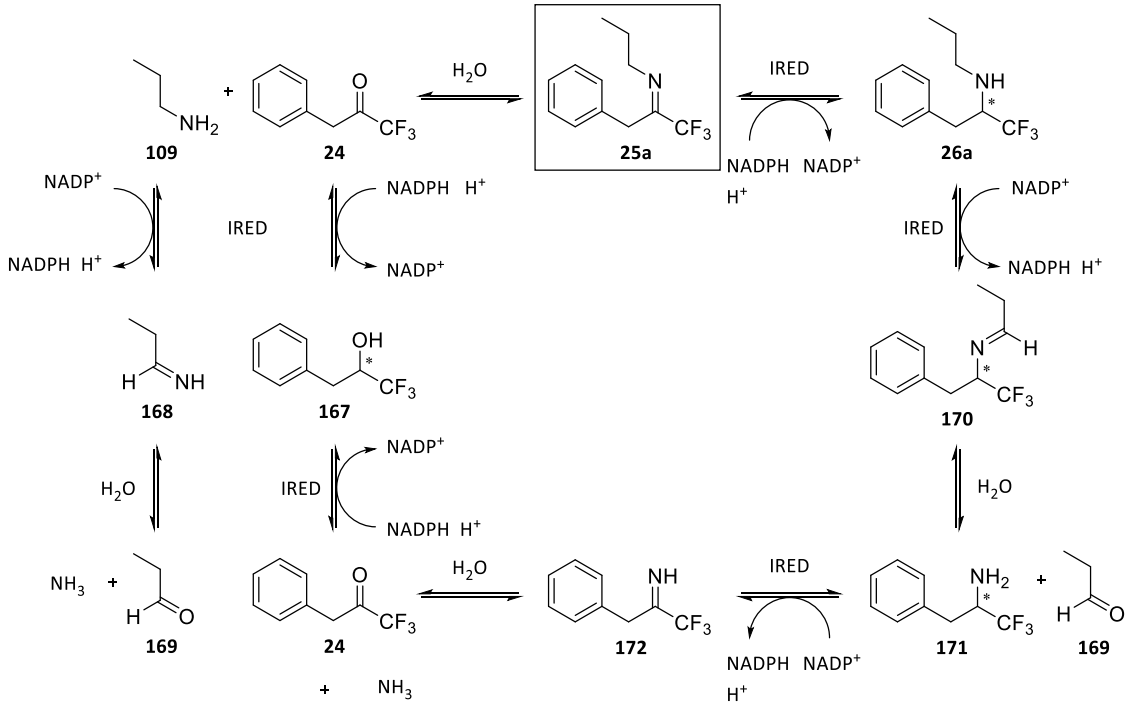


Figure 8. Potential reactions and resulting species that can theoretically occur in the reaction mixture.

Analysis method development

In order to monitor the reaction and study the influence of different reaction conditions, the desired secondary amine **26a**, imine **25a** and possible side products in the reaction mixture must be quantitatively analyzed. A lot of time was spent finding and optimizing a good analysis method. First, this was attempted with reversed phase HPLC with MS (electrospray ionization, 70 eV) and UV detection. To inactivate the enzyme, 500 µL of acetonitrile was added to the 500 µL samples, followed by centrifugation, filtration and injection on the HPLC system. Unfortunately, this method was not sensitive enough for the compounds and concentrations in question. Next, GC-MS (electron impact ionization) was considered as an analysis method, GC-MS was chosen over GC-FID because the MS spectra can be useful for the identification of unknown peaks. A negative aspect of this method is that it is very time-consuming due to the longer analysis time (20 min per sample) and sample preparation. The samples need to be extracted with a volatile solvent that is compatible with GC (CH_2Cl_2). Since amines and imines to a lesser extent are typically basic (CF_3-amines and imines less so) and thus can be protonated at the reaction pH of 7, the samples have to be basified with sodium hydroxide to ensure efficient extraction. The addition of sodium hydroxide also serves as enzyme inactivation. A custom GC-MS method (synthese.HC) with selected ion detection was designed to increase sensitivity and to decrease noise.

In order to determine the aqueous concentrations of the analytes and to improve precision and accuracy of the GC-MS measurements, the use of an internal standard (IS) was opted. A known

amount of IS was added to each sample, immediately after inactivation of the reaction with sodium hydroxide.[68,84,128,131] In this way, the instrumental variabilities (*e.g.* injection volume) and the variability in sample preparation (*e.g.* extraction efficiency) are compensated. If the relative response factors (rRF) of the analytes relative to the internal standard are known, it would in principle be possible to determine the aqueous concentration of each analyte, assuming that they all are extracted with the same efficiency.[68] Therefore the IS should preferably resemble the analytes in physicochemical properties (pKa and lipophilicity), but should also be resolvable from all analytes on the GC column. Amine **26b** was selected as the IS because it only has one extra methylene unit compared to amine **26a** (the most important analyte) and is therefore expected to be extracted from the reaction mixture with nearly identical efficiency. To that end, calibration curves were set up for the three analytes (ketone **24**, amine **26a** and imine **25a**) with in each sample a fixed concentration (2 mM) of the IS **26b**. When constructing the calibration curves, it became clear that the addition of the IS was absolutely necessary due to instrumental variation. This is shown in Figure 9, where the calibration curves normalized to the internal standard (ratio of peak areas versus ratio of concentrations) clearly gave superior results. The possibility of human error in sample preparation for the standard curves was ruled out, because a second analysis of the same samples resulted in a different pattern for the concentration-peak area curves.

The method of determining the aqueous analyte concentrations was abandoned due to unexplainable and illogical results. Instead, it was decided to calculate formations as a percentage of the three analytes. In this method, the instrumental variability is not expected to pose a problem, as only relative amounts of analytes are considered. The calibration curves with the internal standard and the corresponding relative response factors were nonetheless useful for calculating the analyte formations (equation 1).

$$formation(analyte) = \frac{\frac{A(analyte)}{rRF(analyte)}}{\frac{A(amine)}{rRF(amine)} + \frac{A(imine)}{rRF(imine)} + \frac{A(ketone)}{rRF(ketone)}} * 100\% \qquad (1)$$

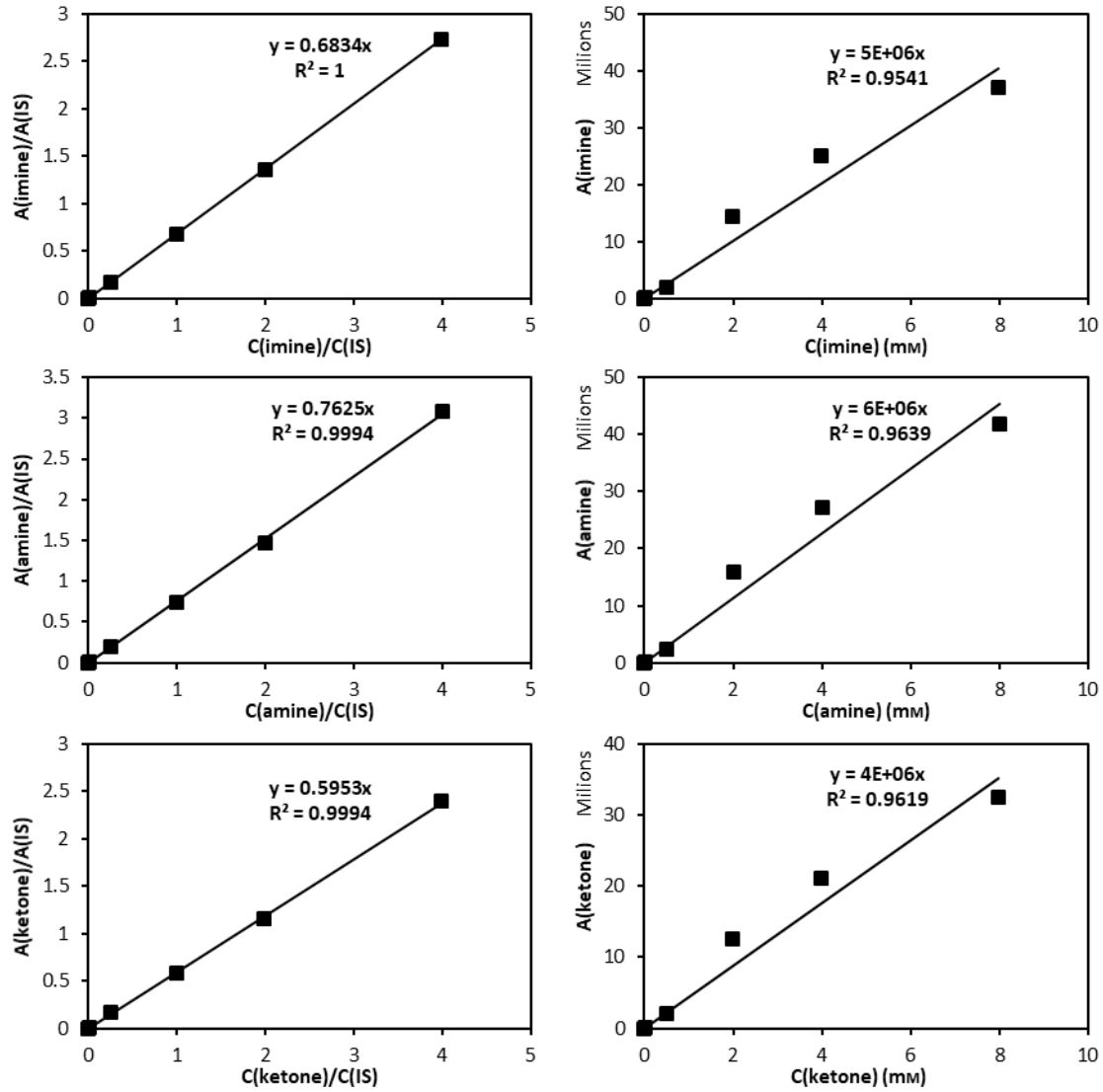


Figure 9. GC-MS calibration curves (selected ion detection) for imine **25a**, amine **26a** and ketone **24**. Left: responses of analytes are normalized to a fixed amount of internal standard **26b**. Right: peak area responses of the analytes versus the concentration in mM. The relative response factors (rRF) were calculated from the graphs on the left as the slopes of the fitted trend lines.

Table 7. GC-MS retention times and relative response factors of analytes relative to the internal standard, amine **26b**.

Analyte	RT (min)[a]	$rRF_{total\ ion}$	$rRF_{selected\ ions}$[b]
Imine **25a**	6.24	0.6807	0.6834
Amine **26a**	6.40	0.6939	0.7625
Ketone **24**	2.88	0.4877	0.5953
Alcohol **167**	4.41	n.d.[c]	n.d.[c]
Internal standard **26b**	7.37	1	1

[a] The retention times of all analytes were shortened by about 0.15 min during the project after a maintenance. The most recent retention times are given.

[b] Only the most abundant fragment ions for these analytes were detected. These were at m/z 43, 91, 98, 112, 118, 140, 154, 160, 188 and 190.

[c] The alcohol side product **167** was not formed in any of the enzymatic reactions, so no calibration curve was made for it.

Results

The enzymatic reduction of imine **25a** with cofactor recycling was studied for the two productive *R*-IREDs and also with *S*-IRED-*Pe,* because it was desirable to obtain both enantiomers of amine **26a**. Multiple 1 mL scale experiments were set up for each enzyme and samples were taken after 16.25 h and 24 h of reaction. Initially it was planned to study the influence of substrate concentration (5, 10 and 15 mM). Unfortunately, the imine stocks for the experiments with 5 and 15 mM **25a** had degraded and there was no time to repeat the experiments due to the COVID-19 crisis. However, the reactions with 10 mM already provided some interesting insights. In the literature, these reactions are sometimes performed in glass vials and sometimes in plastic (polypropylene) Eppendorf tubes. Therefore, both reaction vessel types were used for comparison. Furthermore, the hydrolysis of the imine was studied in an experiment in which no IRED was added. In this experiment, basification with sodium hydroxide was omitted as this might influence the equilibrium concentrations of ketone **24** and imine **25a**. Lastly, the necessity of sodium hydroxide addition to the samples, for amine extraction into the organic phase (CH_2Cl_2), was investigated.

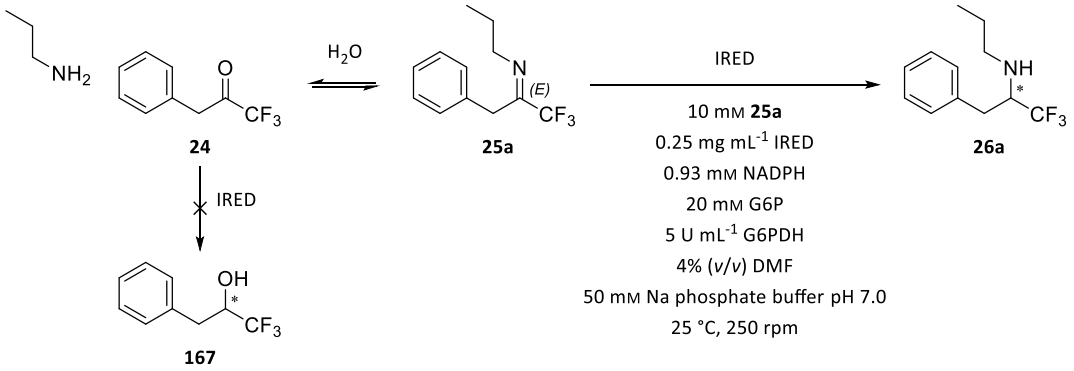
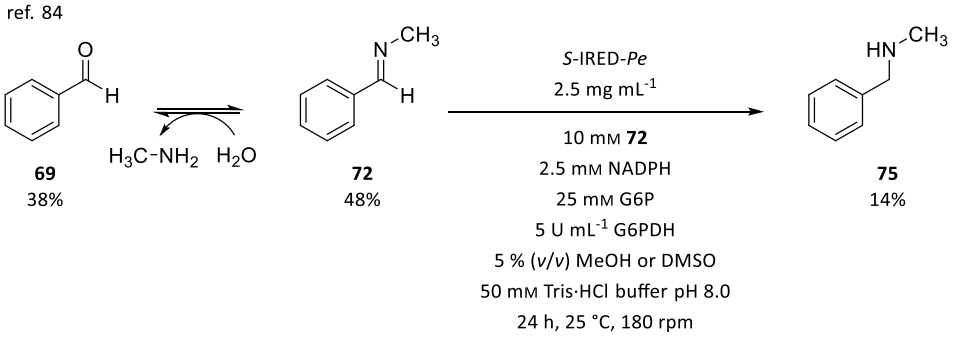


Table 8. Results from the enzymatic reduction of trifluoromethyl imine **25a** with three IREDS and with cofactor regeneration.

	Experiment	Amine 26a (%)		Imine 25a (%)		Ketone 24 (%)	
		16.25 h	24 h	16.25 h	24 h	16.25 h	24 h
	S*-IRED-*Pe						
1	Glass vial	0.4	0.0	98.8	97.5	0.8	2.5
2	Eppendorf	0.8	0.0	97.8	88.3	1.4	11.7
	R*-IRED-*Si						
3	Glass vial	20.0	26.0	79.9	69.0	0.1	5.0
4	Eppendorf	71.0	62.5	25.4	21.1	3.6	16.4
5	Glass vial, no NaOH	16.0	15.4	37.4	23.8	46.6	60.7
	R*-IRED-*Ss						
6	Glass vial	36.0	57.1	63.9	36.3	0.1	6.6
7	Eppendorf	93.3	24.0	5.3	74.4	1.4	1.6
	No IRED (hydrolysis)						
8	Glass vial, no NaOH	0.1	0.0	53.9	54.1	46.1	46.0

Fractions of the analytes were calculated from the GC-MS chromatogram peak areas using equation 1.
Only the most abundant fragment ions for these analytes were detected. These were at m/z 43, 91, 98, 112, 118, 140, 154, 160, 188 and 190.

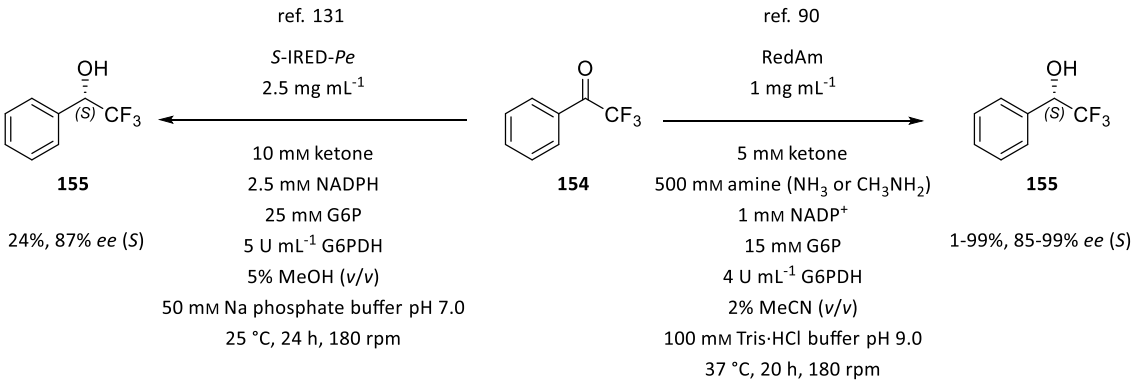
From the results, it is clear that *S*-IRED-*Pe* hardly produced any amine (< 1%). On the contrary, *R*-IRED-*Si* and *R*-IRED-*Ss* did form considerable amounts of amine **26a** in each experiment. The small amount of amine (0.1%) in the hydrolysis experiment (entry 8), is suggested to be a contamination. The most productive IRED in each experiment was *R*-IRED-*Ss*, which formed up to 93.3% amine. In order to increase amine **26a** formation, the enzyme concentration and the cofactor concentration could be raised. *S*-IRED-*Pe* is reported to produce a meager 14% of secondary amine **75** from aldimine **72**, with 2.5 mg mL^{-1} enzyme and 2.5 mM NADPH.[84]



In terms of reaction vessel, it is clear that Eppendorf tubes yield superior results compared to glass vials for both of the active enzymes, at least when considering the conversions after 16.25 h. However, the reactions with the *R*-IREDs in the Eppendorfs (entries 4 and 7) seem to go backwards between the two time points. The amine seemingly gets oxidized back to the imine (amine: 71.0% to 62.5% for *R*-IRED-*Si* and 93.3% to 24.0% for *R*-IRED-*Ss*). In the glass vials, the amine fraction only rises (entries 3 and 6) or remains constant (entry 5). This could imply that there is some oxidative driving force that is only prevalent in the Eppendorf tubes. It is unclear what this driving force could be and why it is more prominent in Eppendorf tubes. In a study about IRED-catalyzed reductive aminations, the autoxidation of NADPH by molecular oxygen was mentioned as a potential pathway for $NADP^+$ formation.[99] To prevent the oxidation of the formed amine, the reaction should be stopped in time. It may also be helpful to add more equivalents of the glucose-6-phosphate reductant. This apparent oxidation activity does raise the question of how many potential species depicted in Figure 8 may be present in the reaction mixture. It is also not clear why the reactions in the Eppendorf tubes would result in more product formation after 16.25 h. This experiment should be repeated with more frequent sampling (*e.g.* 1 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h) in order to understand the dynamic processes that are taking place. The failed experiments with varying imine **25a** concentrations should also be repeated for optimization of the productivity. Lastly, reactions should ideally be performed in triplicate to ensure repeatability, this could not be done within the time frame of this project.

A basic pH is known to favor imine formation in aqueous environment (see section 3.2.4.1 Imine formation). Therefore, adding such a large amount of sodium hydroxide (final concentration 0.57 M) is expected to significantly skew the equilibrium in the sample towards imine **25a**, resulting in an inaccurate representation of the actual reaction.[83] Comparison of the experiments with (entries 1, 2, 3, 4, 6 and 7) and without basification (entries 5 and 8) reveals that sodium hydroxide addition does result in an underestimation of hydrolysis. However, it is also clear (entries 3 and 5) that some basification is necessary to ensure efficient extraction of amine **26a** into the dichloromethane phase. In future research, it might be better to significantly lower the amount of sodium hydroxide added or to use a milder base (*e.g.* $NaHCO_3$), so that extraction remains efficient, while minimizing imine formation during sampling.

In future research, this pH effect on the imine-ketone equilibrium might be put to good use in the reaction set up.[84] After all, it is already widely applied in IRED-catalyzed reductive aminations. The hydrolysis experiment shows that imine **25a** undergoes about 46% hydrolysis, which is less than expected. After all, attempts in the literature of reductive aminations of phenyl trifluoromethyl ketone **154**, in the presence of 50-fold excess primary amine and at basic pH, were unsuccessful due to no or little imine formation. If a basic pH (8 - 9) is applied in the enzymatic reaction, hydrolysis would be less favored and therefore imine reduction would be kinetically favored. However, the enzyme kinetics and stability may also suffer from a higher pH. Furthermore, the imine-ketone equilibrium may also be shifted toward the imine by addition of propylamine. Having said that, imine **25a** is clearly present at sufficiently high concentrations (~ 5 mM) for enzymatic reduction to occur. The observed hydrolytic stability of imine **25a** might be the result of the electron-withdrawing CF_3 substituent on the imine carbon.[169]



Surprisingly, the formation of alcohol **167** was not detected in any of the experiments. This is a very promising result, because the (reductive) stability of ketone **24** is crucial for obtaining potential full conversion of the substrate imine **25a**. Past attempts to produce chiral trifluoromethyl amines with IREDs, *via* reductive amination of ketone **154** that is, have resulted

in exclusive alcohol **155** formation.[90] Furthermore, *S*-IRED-*Pe* has been reported to reduce the same ketone **154**. It was therefore expected that at least some ketone **24** reduction would occur with this enzyme.

In conclusion, the first successful enzymatic reduction of a trifluoromethyl imine using IREDs was achieved. Conversions of up to 93.3% and 71.0% were obtained with *R*-IRED-*Ss* and *R*-IRED-*Si*, respectively.

3.2.3 Enantioselectivity of IREDs for the formation of trifluoromethyl amines

The enantiomeric purity of bioactive molecules like pharmaceuticals is often crucial for their safe use, as both enantiomers can have vastly different biological activities (see section 1 Background and goal).

Having achieved considerable product formation in 1 mL scale reactions, the next planned step was the determination of the enantioselectivity of these enzymes. This includes analytical resolution of the two formed enantiomers, *e.g. via* chiral chromatography, and subsequent calculation of the enantiomeric excess. Next, the absolute configuration of the major enantiomer of amine must be determined to find out the enantiopreference of the IREDs. Due to the COVID-19 crisis, the lab work was cut short, so experiments for this part were very limited. Therefore, this section serves mainly as a guideline for future research.

3.2.3.1 Enantiomeric excess determination

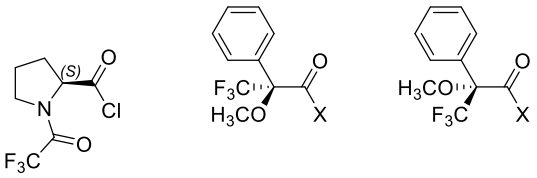
The enantiomeric excess (*ee*) is defined as the difference between the mole fraction of the major enantiomer (F(M)) and the mole fraction of the minor enantiomer (F(m)). These fractions may be measured when the product is exposed to some kind of chiral environment. This can be a chiral non-racemic solvent, solvating agent or derivatizing agent, or a chiral stationary phase, or circularly polarized light. The mole fractions can then be determined by measuring the different peak areas (A) in a chromatogram or spectrum.[170]

$$ee(\%) = [F(M) - F(m)] * 100\% = \frac{A(M) - A(m)}{A(M) + A(m)} * 100\% \qquad (2)$$

First a normal phase chiral HPLC method was considered, since this would allow the direct use of the GC-MS samples from the 1 mL scale reactions (see above). To that end, a racemic mixture of amine **26a**, obtained *via* reduction of imine **25a** with lithium aluminum hydride (see section 3.2.1 Chemical, non-stereoselective reduction of trifluoromethyl imines), was analyzed with a chiral HPLC method inspired by the literature.[171] Multiple solvent programs with a normal phase column were attempted, but unfortunately the enantiomers could not be separated. Some experiments

with a reversed phase column were planned, but there was no time to perform them due to the COVID-19 crisis.

Another method that could be considered is the derivatization of amine **26a** with a chiral and enantiomerically pure carboxylic acid or carboxylic acid derivative, resulting in a mixture of diastereomeric amides. As diastereomers have different physical properties in achiral environments, they should be resolvable by conventional analysis techniques like ^{19}F NMR (in case of a preparative scale reaction) or GC-MS. Examples of possible chiral derivatizing agents include *N*-trifluoroacetyl-*L*-prolyl chloride (*L*-TPC) **173** as well as the so-called Mosher's acids (*R*)- or (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) **174** and the corresponding Mosher's acid chlorides (*S*)- or (*R*)-MTPA-Cl **175**.[71,172–175] This method, combined with NMR spectroscopy could also be used for the determination of the absolute configuration of amine **26a** as will be discussed in the next paragraph.[71,176,177] Additionally, chiral carboxylic acids like (*R*)- or (*S*)-MTPA **174** can also be used as chiral solvating reagents for resolution of amine **26a** enantiomers with NMR spectroscopy.[178]



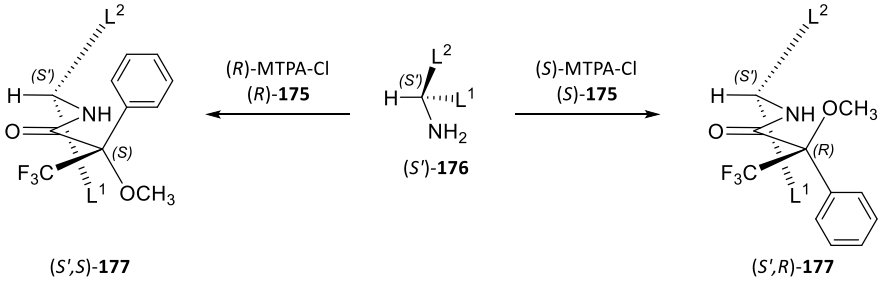
(*S*)-**173** (*S*)-**174** X = OH (*R*)-**174** X = OH
(*R*)-**175** X = Cl (*S*)-**175** X = Cl

3.2.3.2 Absolute configuration determination using Mosher's amides

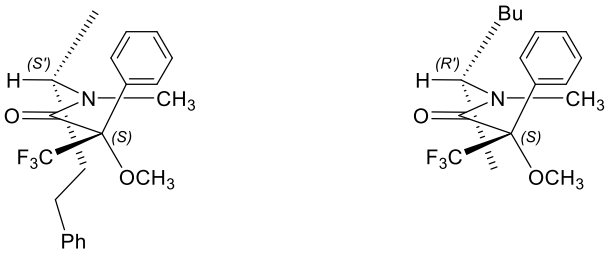
The (enantioselective) synthesis of amine **26a** has not been reported. Therefore, the enantiopreference of the IREDs toward trifluoromethyl imine **25a** cannot be deduced by comparing experimental data of amine **26a** like optical rotation or chiral HPLC elution order with reported data. Instead, the absolute configuration of the major enantiomer of amine **26a** must be determined from scratch. This in turn requires some isolated amine **26a** (~ 50 mg) from a preparative enzymatic reaction. The stereopreference of the other IREDs can then be deduced by comparing the chiral HPLC chromatograms. Due to time constraints, a preparative reaction could not be performed. However, an effort was made to develop a method to determine the absolute configuration of amine **26a** using Mosher's method.[177,179]

This method typically involves the following steps. A chiral, usually primary amine **176** with unknown absolute configuration (for the sake of the example let's assume that it is (*S'*)-configured) is derivatized with both (*R*)- and (*S*)-MTPA-Cl **175** (caution, (*R*)-MTPA-Cl yields (*S*)-MPTA-amide). The ^{1}H and ^{19}F NMR spectra of the resulting pair of amide diastereomers **177** are

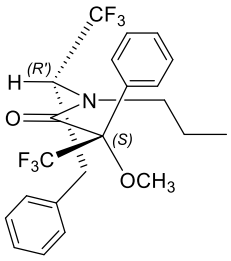
then compared. According to Mosher's model, the minimal energy conformations of the amides look like the ones depicted below. In ^{1}H NMR experiments, the nuclei near the investigated stereogenic center (the nuclei on L^1 and L^2) will have different shifts in the two diastereomers due to anisotropic shielding by the phenyl ring. With the knowledge of the configuration of the used MTPA-Cl **175** agents, it is possible to deduce the configuration of (*S'*)-**176**.[179,180]



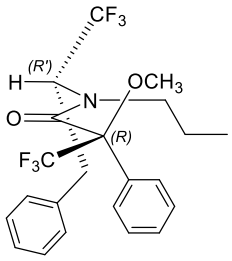
For secondary amines like **26a**, the situation becomes more complicated. The resulting tertiary Mosher's amides **180** will be present in solution as a mixture of rotamers *syn*-**180** and *anti*-**180** due to hindered rotation around the amide C-N bond.[71,82,179] A database analysis of crystal structures of 17 tertiary Mosher's amides derived from chiral secondary amines revealed that the vast majority of these amides are in the *anti*-conformation.[181] This analysis did not specify whether the methine group was *synplanar* with the carbonyl as is the case for secondary Mosher's amides **177**. However, reports of tertiary amides (*S'*,*S*)-**178** and (*R'*,*S*)-**179** are consistent with this conformation.[71,82]



Based on all these facts, an educated guess was made that the Mosher's amide derivatives **180** of amine **26a** might also exists in this conformation. The plausible minimal energy conformations of both MTPA-Cl derivatives of amine (*R'*)-**26a** are displayed in the scheme below as an illustration.



anti-(*R*',*S*)-**180**
major rotamer



anti-(*R*',*R*)-**180**
major rotamer

To confirm or disprove this plausible conformation, it was planned to synthesize both possible diastereomers of **180** from the racemic amine **26a** and one of the MTPA-Cl derivatizing agents **175**. A separation of the diastereomers would be attempted by crystallization or by chromatography. Subsequently, both diastereomers would be characterized in terms of their crystal structure (X-ray) and ^{1}H and ^{19}F NMR spectra. Unfortunately, no formation of amides **180** was detected and MTPA-Cl **175** was hydrolyzed. It was argued that the electron-withdrawing effect of the trifluoromethyl group on amine **26a** might decrease its nucleophilicity. If there was more time in this project, the following adaptations to the reaction conditions would have been tried. To the mixture of unreacted amine **26a** and hydrolyzed MTPA-Cl (carboxylic acid) **174**, could be added the coupling reagent *N*,*N*'-dicyclohexylcarbodiimide (DCC) **181**. These conditions have been reported for the MTPA-Cl derivatization of α-trifluoromethyl primary amine **171**. However, for secondary amine **26a**, this reaction might be hampered due to steric hindrance from the *n*-propyl group.[175] Furthermore, the reaction with MTPA-Cl **175** could be modified by the addition of nucleophilic catalysts like 4-dimethylaminopyridine (DMAP) **182** or 4-pyrrolidinopyridine (PYP) **183**.[182] If the reaction still does not proceed under these conditions, a strong base like sodium hydride, lithium diisopropylamide (LDA) or *tert*-butyllithium might be used for deprotonation of amine **26a**, followed by addition of the MTPA-Cl **175**.

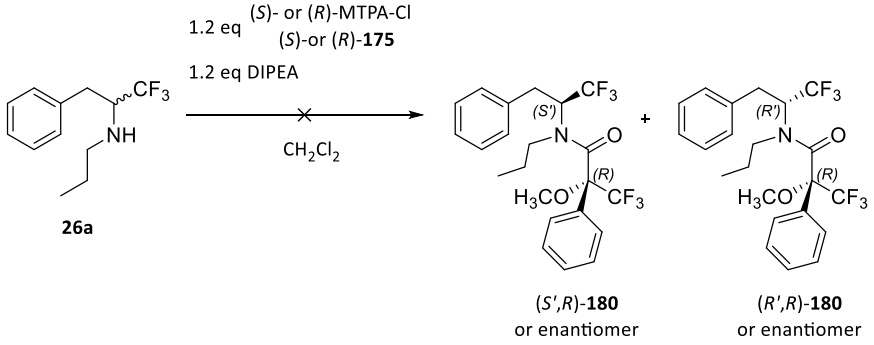
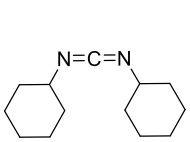


Table 9. Failed synthesis of MTPA-Cl derivatives **180**.

Compound	Acid chloride[c]	Conditions	Hydrolysis (%)[a]	Conversion (%)[a]
180	(*S*)-**175**	rt, 5 h	100	0
180	(*R*)-**175**	rt, 3 h	63[b]	0

[a] ^{19}F NMR analysis in $CDCl_3$.
[b] Extra care was taken to ensure that the reaction was dry.
[c] The stereochemistry of MTPA-Cl **175** does not influence the reaction.



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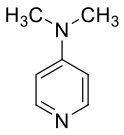

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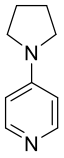
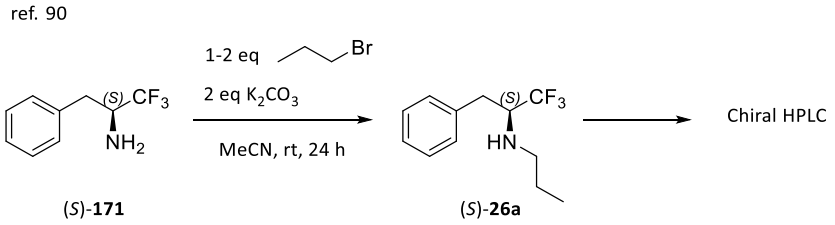


183

Another method for determining the absolute configuration is to buy the commercially available enantiopure product standard of primary amine (*S*)-**171** with known absolute configuration. This standard can be alkylated to make secondary amine (*S*)-**26a**.[90,183] The chiral HPLC retention time of amine (*S*)-**26a** could then be compared with the samples from the enzymatic reductions to determine the enantiopreference of the IREDs.



(*S*)-**171** (*S*)-**26a**

3.3 Conclusions and future perspectives

Amine **26a** was produced in high conversion by two *R*-selective imine reductases, *R*-IRED-*Si* and *R*-IRED-*Ss*, *via* reduction of trifluoromethyl imine **25a**. Organic synthesis was thus successfully combined with biocatalysis. This is the first time that an α-trifluoromethyl amine was successfully produced *via* enzymatic imine reduction. Some hydrolysis and associated ketone **24** formation was observed, however, the imine **25a** concentration seems to remain high enough for the imine reduction reaction to continue. This is quite interesting, since attempts of reductive aminations of phenyl trifluoromethyl ketone **154** with a large excess of primary amine and at basic pH in the literature were unsuccessful due to no or little imine formation. No alcohol **167** formation through promiscuous ketone reduction was detected. This was surprising, since α-trifluoromethyl ketones are quite reactive and their IRED-catalyzed reductions are reported in the literature.

In future research, the imine reduction experiments may be adapted as discussed above. Aside from imine reductions, it would be very interesting to investigate the possibility of reductive

amination. If ketone **24** could be combined with primary or even secondary amines in an aqueous buffer and be reductively aminated using IREDs, then a workup step can be omitted. This could significantly improve the yield and productivity of this transformation. The high equilibrium imine **25a** concentrations in the aqueous buffer are encouraging to attempt this route. Lastly, the rationale for the determination of the absolute configuration of amine products was also developed to facilitate future research.

4 Summary and conclusion

Chiral α-trifluoromethyl amines, and specifically amphetamine derivatives like trifluorinated selegiline derivative **27a**, are potentially interesting compounds for medicinal chemistry. Chemical, enantioselective synthesis methods for these types of compounds are inherently dangerous, difficult and potentially harmful for the environment. Enzymes represent a greener alternative to the chemical methods as they operate in aqueous buffers, under mild conditions and are often very selective. Moreover, the disposal of these biocatalysts is also harmless, unlike transition metal complexes and other typical chemicals used for synthesis.[30,32]

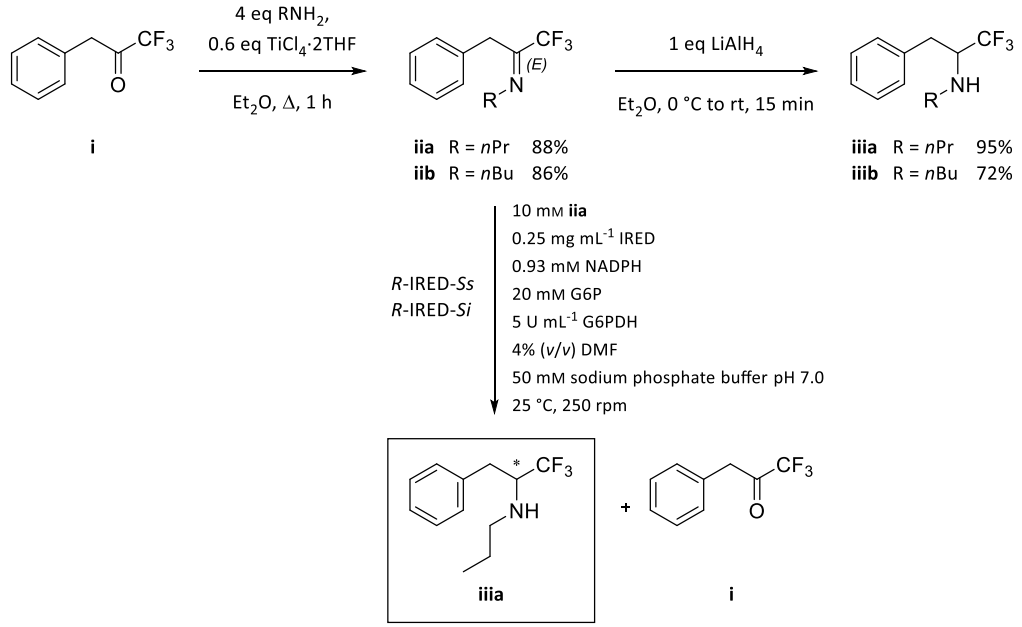
Imine reductases (IREDs) and their subclass, reductive aminases (RedAms), are a fairly new class of enzymes which catalyze the enantioselective, NADPH-dependent reduction of imines and iminium ions. These enzymes have been under investigation for the asymmetric synthesis of chiral primary, secondary and tertiary amines. Several valuable and biologically active amines have been successfully prepared using these enzymes.

4.1 Summary

In the initial phase of this Master's thesis, (*E*)-trifluoromethyl imines **iia** and **iib** were prepared *via* condensation of 1,1,1-trifluoro-3-phenylpropan-2-one **i** with the corresponding primary amines. The *E*-stereochemistry of the imines was determined through a combination of 2D NOESY experiments and molecular mechanics simulations. Hereafter, amines **iiia** and **iiib** were prepared *via* lithium aluminum hydride-mediated reduction of imines **iia** and **iib**, respectively.

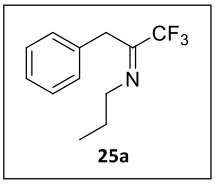
In a second phase, the enzymatic reduction of imine **iia** with *S*-IRED-*Pe*, *S*-IRED-*Ss*, *R*-IRED-*Si* and *R*-IRED-*Ss* was investigated. Only the *R*-selective IREDs succeeded at reducing imine **iia** and *R*-IRED-*Ss* was clearly the most productive of the two, achieving an impressive 93% conversion. Fortunately, none of the studied IREDs displayed promiscuous ketone reduction activity. The influence of factors like reaction vessel type and sodium hydroxide addition were also studied. It was concluded that Eppendorf tubes are superior to glass LC vials as reaction vessels, because the former reactions reached the best conversions after 16.25 h. However, after 24 h the reactions in the Eppendorf tubes seemingly reverted with oxidation of amine **iiia** to imine **iia**. Furthermore, it was clear from the GC-MS results that the addition of sodium hydroxide during the sampling procedure causes an underestimation of the imine hydrolysis. However, this is not a big problem, since the knowledge of imine **iia** concentration is of secondary importance to the amine **iiia** concentration. The latter could only be accurately measured if basification with sodium hydroxide was used.

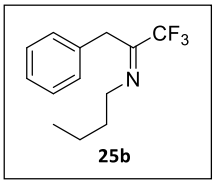
Lastly, a method was developed for the determination of the stereochemistry of the enzymatically formed amine **iiia**, based on an inquiry into the literature. This method could not be validated within the time frame of this project, but can serve as a guideline for future research.

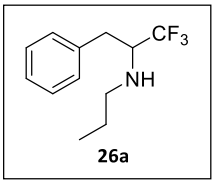


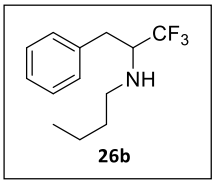
4.2 Conclusion

A preliminary investigation into the potential of imine reductase-mediated synthesis of trifluorinated amphetamine derivatives **26** was successfully performed. This is the first successful example of an enzymatic trifluoromethyl imine reduction. Although no product could be isolated on a preparative scale and the stereoselectivity could not be determined, product formation of up to 93% was achieved, providing a suitable basis for follow-up research in the future. Thus, classical organic synthesis was successfully combined with biocatalysis, which is an important part of the green chemistry concept.





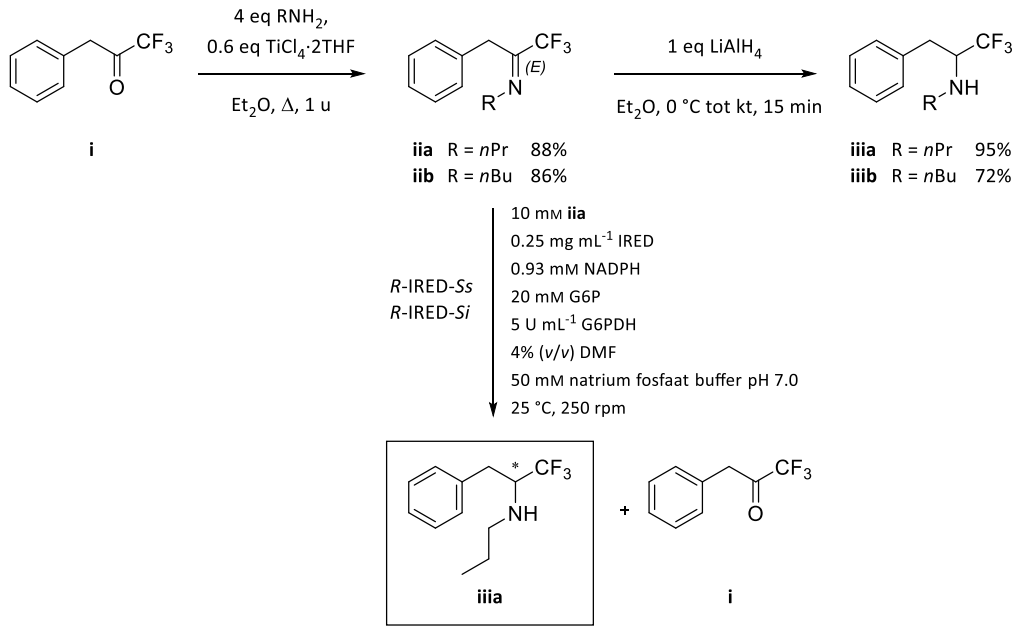




5 Samenvatting

Chirale α-trifluormethylaminen en meerbepaald amfetaminederivaten zoals trigefluoreerd selegiline derivaat **27a**, zijn potentieel interessante verbindingen voor de medicinale chemie. Chemische, enantioselectieve synthesemethoden voor dit type verbindingen zijn inherent gevaarlijk, moeilijk en zijn potentieel schadelijk voor het milieu. Enzymen zijn een groener alternatief voor deze chemische methoden, aangezien ze in waterige bufferoplossingen werkzaam zijn, bij milde reactieomstandigheden en bovendien bijzonder selectief zijn. Daarboven kunnen deze biokatalysatoren zonder probleem weggeworpen worden na gebruik, aangezien ze gewoonlijk niet schadelijk of toxisch zijn. Dit in tegenstelling tot transitiemetaalcomplexen en andere conventionele chemicaliën die in synthesen worden aangewend.[30,32]

Imine reductasen (IREDs) en hun subklasse, reductieve aminasen (RedAms) zijn een tamelijk nieuwe enzymklasse die de enantioselectieve, NADPH-afhankelijke reductie van iminen en iminiumionen katalyseren. Deze enzymen zijn reeds onderzocht voor de asymmetrische synthese van chirale primaire, secundaire en tertiaire aminen. Verscheidene waardevolle en biologisch actieve aminen zijn reeds met succes bereid met deze enzymen. De IRED-gekatalyseerde reductie van (trifluormethyl)benzylketiminen **ii** is bijgevolg een veelbelovende synthesemethode voor optisch zuivere trigefluoreerde amfetamine derivaten **iii**.



In dat opzicht werden in de eerste fase van deze Masterproef (*E*)-trifluormethyliminen **iia** en **iib** bereid *via* condensatie van 1,1,1-trifluor-3-fenylpropan-2-on **i** met de overeenkomstige primaire aminen. Vervolgens werden aminen **iiia** en **iiib** bereid door lithiumaluminiumhydride-gemedieerde reductie van respectievelijk iminen **iia** en **iib**.

In een tweede fase werd de enzymatische reductie van imine **iia** met *S*-IRED-*Pe*, *S*-IRED-*Ss*, *R*-IRED-*Si* en *R*-IRED-*Ss* onderzocht. Enkel de *R*-selectieve IREDs konden imine **iia** met succes reduceren en *R*-IRED-*Ss* was duidelijk het productiefste enzym, met bereikte omzettingen tot 93%. Dit is het eerste voorbeeld van een geslaagde enzymatische reductie van een trifluormethylimine. Er kon echter binnen het tijdsbestek van deze Masterproef geen preparatieve enzymatische reductie uitgevoerd worden.

De enantioselectiviteit van deze reacties kon overigens niet bepaald worden binnen het tijdsbestek van deze Masterproef. Er werd echter wel een methode opgesteld om de absolute configuratie van amine **iia** te bepalen na het bestuderen van de relevante literatuur. Deze methode kon niet gevalideerd worden, maar kan dienen als een leidraad voor toekomstig onderzoek.

6 Experimental part

6.1 Reagents and solvents

All chemicals were purchased from commercial suppliers and used without further purification. Diethyl ether (Et_2O), toluene and dichloromethane (CH_2Cl_2) were dried with the MBRAUN SPS-800 solvent purification system. The used imine reductases ware purchased from Enzymicals AG.

6.2 General analytical methods and instrumentation

6.2.1 Thin layer chromatography (TLC)

The determination of R_f-values (retention factors) and the analysis of column chromatography eluent fractions were performed by means of thin layer chromatography. The R_f-values of the synthesized compounds were used for selection of an appropriate solvent mixture for automated and manual column chromatography. Glass-backed silica plates (Merck Silica gel 60 F_{254}, precoated, thickness 0.25 mm) were used in combination with an experimentally determined solvent mixture. Visualization of the analyte spots was achieved with UV irradiation (254 nm) combined with a potassium permanganate stain with subsequent heat treatment.

6.2.2 Column chromatography

For preparative purification by means of column chromatography, silica gel was used as a stationary phase (particle diameter 35-70 µm, pore diameter 6 nm). An appropriate solvent mixture was identified with TLC. Depending on the amount of product, a glass column with a suitable diameter was selected. The elution speed was approximately 5 cm min^{-1}.

6.2.3 Automated column chromatography

Preparative purification was carried out using automated column chromatography with a Grace Reveleris™ Flash Chromatography system (SiO_2). To that end, reusable columns (SiO_2, particle diameter 0.040-0.063 mm) were employed. The elution speed was dependent on the column size. Detection of the compounds was performed *via* UV-detection at three selected wavelengths and *via* ELSD-detection (Evaporative Light Scattering Detector).

6.2.4 High performance liquid chromatography mass spectrometry (HPLC-MS)

Liquid chromatography mass spectrometry was employed for the monitoring of reactions and for analysis of crude reaction mixtures. These analyses were performed with an Agilent 1200 Series LC-MSD SL device with a Supelco Ascentis® Express C18 column (I.D. x 4.6 mm x 3 cm, 2.7 µm

fused core particles with 90 Å pore size). For the detection of analytes, this device is equipped with a UV-DAD detector, an Agilent 1100 Series MSD SL mass spectrometer with electrospray ionization (ESI, 4000 V, 70 eV) and a single quadrupole detector. A solvent mixture of acetonitrile and water (5 mM ammonium acetate) was used as mobile phase. The ratio acetonitrile to water varied depending on the selected method.

6.2.5 Chiral HPLC

Enantiomeric excess values were determined via chiral HPLC analysis using an Agilent 1200 series LC/MSD SL-system, equipped with a UV detector and a Daicel ChiralPak® IA column (amylose tris(3,5-dimethylphenylcarbamate) immobilized on 5 µm silica gel, I.D. x L 4.6 mm x 250 mm) at a column temperature of 30–35 °C using flow rates of 0.3–1 mL/min. The detection wavelength was set at 204.0, 210.0 and 214.0 nm.

6.2.6 Gas chromatography mass spectrometry (GC-MS)

Gas chromatography mass spectroscopy was employed to characterize synthesized products and to monitor the enzymatic imine reduction reactions. To this end, samples, dissolved in dichloromethane (CH_2Cl_2), were analyzed with an Agilent 6890A gas chromatography device equipped with an Agilent J&W DB-5MS column (30 m x 0.25 mm x 0.25 µm). Samples (10 µL) were injected with a split inlet ratio 10:1, inlet pressure 78.5 kPa, inlet temperature 250 °C, linear velocity 40 cm sec^{-1}, carrier gas Helium. Temperature program: 80 °C, 10 °C min^{-1} to 200 °C, 30 °C min^{-1} to 280 °C, hold 5 min. Detection was performed with an Agilent HP 5973 Series MSD mass spectrometer with electron impact ionization (IE). For some of the analyses a specialized ion detection method was used (m/z 43, 91, 98, 112, 118, 140, 154, 160, 188 and 190).

6.2.7 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR, ^{19}F NMR and ^{13}C NMR spectroscopy were employed for the monitoring of reactions, for the analysis of crude reaction mixtures, and for the characterization of isolated compounds. Spectra were recorded using a Bruker Avance Nanobay III NMR spectrometer, equipped with ^{1}H/BB z-gradient high resolution probe (BBO, 5 mm). ^{1}H NMR, ^{19}F NMR and ^{13}C NMR spectra were recorded at 25 °C at 400, 376 and 100 MHz using the standard sequences in the Bruker pulse program library. The samples were dissolved in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as internal standard. ^{1}H and ^{13}C chemical shifts (δ) are reported in parts per million (ppm) downfield of TMS and referenced to the residual solvent peak ($CDCl_3$ δ_H = 7.26, δ_C = 77.16). ^{19}F chemical shifts are uncorrected. All spectra were processed using TOPSPIN 3.2. Coupling constants (*J*) are reported in hertz (Hz). Peaks were assigned with the aid of 2D spectra

(COSY, HSQC, HMBC) and stereochemistry suggestions were made by means of 2D NOESY experiments.

6.2.8 Mass spectrometry (MS)

Mass spectrometry (low resolution) was used for the characterization of the synthesized compounds. These analyses were performed using an Agilent 1100 Series MSD SL mass spectrometer with electrospray ionization (ESI, 4000 V, 70 eV) with a single quadrupole detector.

6.2.9 Infrared spectroscopy (IR)

Infrared spectroscopy of synthesized compounds was performed with a Shimadzu IRAffinity-1S device with an Attenuated Total Reflectance (ATR) crystal. The measurements were processed by the LabSolutions IR software. Only selected absorbances (ν_{max}) are reported in cm^{-1}.

6.2.10 Spectrophotometry

Protein concentration and enzyme activity determinations were performed by measuring the absorbance at 562 and 340 nm, respectively, with a 680XR microplate reader spectrophotometer (Bio-Rad).

6.2.11 Computational methods

The *E*/*Z* configuration of imines **25** were determined using a combination of 2D NOESY NMR spectroscopy and molecular modelling. The minimal energy conformations of imines **25** were simulated using the Molecular mechanics (MM2) tool of the Chem3D Pro 16.0 software (CambridgeSoft, PerkinElmer Informatics, Waltham, MA, USA), using standard settings (minimize energy to minimum RMS gradient of 0.010).

6.3 Safety

6.3.1 General safety aspects

All experiments in this Master's thesis were performed in compliance with the internal safety guidelines of the SynBioC Research Group (Department of Green Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University), 'Veiligheid en hygiene in chemische laboratoria', 'Safety instructions: how to work with chemicals' and 'Welzijns- en Milieugids UGent'.[184–187] These documents were thoroughly read before the start of the laboratory activities. Before any new experiment was executed, the Safety Data Sheets (SDS) of the used substances were consulted. Furthermore, extra care was taken in handling dangerous reagents or using hazardous equipment in order to ensure personal protection as well as the safety of bystanders.

6.3.2 Specific safety aspects

During the experimental work for this project, the use of dangerous reagents was avoided to minimize any safety risks. However, in some instances there was no safe alternative. An overview of the most important dangerous chemicals along with their health and environmental hazards is given.

Silica gel (SiO_2) is used as stationary phase as well as carrier material chromatography applications. Due to the small particle size, inhalation must be avoided by wearing a dust mask.

Sodium hydroxide (NaOH) (10 M) causes severe skin burns and eye damage.

Liquid nitrogen is used for the high vacuum installation. Can cause severe cryogenic burns to the skin and eyes. May cause suffocation by displacing oxygen in the air.

Toluene, hexane, petroleum ether, diethyl ether are highly flammable; keep away from ignition sources. Inhalation of vapors may be dangerous.

2-Methyl-1-pyrroline may cause dizziness, suffocation or delayed pulmonary edema when inhaled. Skin contact may cause cyanosis of the extremities. Eye contact may result in conjunctivitis and corneal damage.

***N*,*N*-Dimethylformamide (DMF)** may damage the unborn child.

Dichloromethane (CH_2Cl_2) causes immediate necrosis of surrounding muscle and skin tissue when accidentally injected. Causes severe eye irritation. May cause cancer.

Deuterated chloroform ($CDCl_3$) causes severe eye irritation and is suspected to cause cancer and to harm the unborn child. Prolonged or repeated exposure may cause damage to the liver and kidneys.

1,1,1-Trifluoro-3-phenylpropan-2-one. Highly Flammable liquid and vapor. Causes skin irritation and severe eye damage. May cause respiratory irritation. Toxic in case of skin contact.

Propylamine and butylamine. Highly flammable liquid and vapor. Causes severe skin burns and eye damage. Is toxic in case of skin contact or inhalation.

***N*,*N*-Diisopropylethylamine.** Highly flammable liquid and vapor. Causes severe eye damage.

α-Methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) releases HCl vapors when contacted with water. Causes severe skin burns and eye damage. Combustible liquid.

Titanium tetrachloride tetrahydrofuran complex ($TiCl_4$·2THF). Flammable solid. Causes severe skin burns and eye damage. May cause respiratory irritation.

Sodium borohydride ($NaBH_4$). When exposed to moisture, acid or high temperature, this substance releases flammable gasses which may ignite spontaneously.

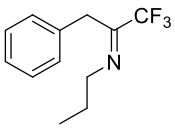
Lithium aluminum hydride ($LiAlH_4$) 1 M solution in THF. When contacted with moisture, this substance releases flammable gasses which may ignite spontaneously. Keep away from ignition sources. Causes severe eye damage. Suspected of causing cancer.

6.4 Description of the experiments

6.4.1 Synthesis of trifluoromethyl imines 25

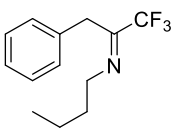
The synthesis of (*E*)-1,1,1-trifluoro-3-phenyl-*N*-propylpropan-2-imine **25a** is described as a representative example. This synthesis and workup procedure were adapted from a PhD thesis conducted at SynBioC.[188] To a solution of 1,1,1-trifluoro-3-phenylpropan-2-one **24** (1.5 mmol) and propylamine (6 mmol, 4 eq) in dry diethyl ether (5 mL), was added $TiCl_4$·2THF (300 mg, 0.9 mmol, 0.6 eq). After one hour of reaction under reflux, the reaction mixture was filtered over a pad of Celite® and washed with diethyl ether (2 x 10 mL). Evaporation of the solvent under reduced pressure afforded (*E*)-1,1,1-trifluoro-3-phenyl-*N*-propylpropan-2-imine **25a** (88%).

(*E*)-1,1,1-Trifluoro-3-phenyl-*N*-propylpropan-2-imine 25a



Orange oil. Yield: 88%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 0.92 (3H, t, *J* = 7.4 Hz, CH_3); 1.69 (2H, ~sextet, *J* = 7.2 Hz, $C\underline{H}_2CH_3$); 3.46 (2H, t, *J* = 7.0 Hz, NCH_2); 3.81 (2H, s, CH_2C=N); 7.15 (2H, d, *J* = 7.4 Hz, 2 x $CH_{arom,ortho}$); 7.24-7.27 (1H, m, $CH_{arom,para}$); 7.30-7.34 (2H, m, 2 x $CH_{arom,meta}$). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 11.8 (CH_3); 23.3 ($\underline{C}H_2CH_3$); 33.0 ($\underline{C}H_2C$=N); 53.8 (CH_2N); 119.9 (q, *J* = 279.5 Hz, CF_3); 127.0 ($HC_{arom,para}$); 128.2 (2 x $HC_{arom,ortho}$); 128.9 (2 x $HC_{arom,meta}$); 134.0 ($C_{arom,quat}$); 157.0 (q, *J* = 32.2 Hz, C=N). **^{19}F NMR** (376 MHz, $CDCl_3$): δ -72.4 (3F, s, CF_3). **IR** (ATR, cm^{-1}): $\nu_{C=N}$ = 1680; ν_{max} = 1198, 1177, 1126, 1080, 733, 704, 694, 642. **MS** (70 eV): m/z (%) 230 ($[M+H]^+$, 100). **GC-MS** (EI): m/z (%) 229 (M^+, 21), 200 (24), 160 (43), 118 (60), 91 (100), 65 (15), 43 (49), 41 (19).

(*E*)-*N*-Butyl-1,1,1-trifluoro-3-phenylpropan-2-imine 25b



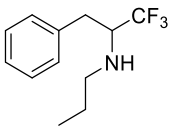
Orange oil. Yield: 86%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 0.90 (3H, t, *J* = 7.3 Hz, CH_3); 1.34 (2H, ~sextet, *J* = 7.5 Hz, $C\underline{H}_2CH_3$); 1.63 (2H, ~quintet, *J* = 7.4 Hz, $NCH_2C\underline{H}_2$); 3.49 (2H, t, *J* = 7.0 Hz, NCH_2); 3.81 (2H, s, CH_2C=N); 7.14 (2H, d, *J* = 7.5 Hz, 2 x $CH_{arom,ortho}$); 7.24-7.27 (1H, m, $CH_{arom,para}$); 7.30-7.34 (2H, m, 2 x $CH_{arom,meta}$). **^{13}C NMR** (100 MHz,

$CDCl_3$): δ 13.8 (CH_3); 20.5 ($\underline{C}H_2CH_3$); 32.1 ($NCH_2\underline{C}H_2$); 33.0 ($\underline{C}H_2C{=}N$); 51.9 (CH_2N); 119.9 (q, *J* = 279.1 Hz, CF_3); 127.0 ($HC_{arom,para}$); 128.2 (2 x $HC_{arom,ortho}$); 128.9 (2 x $HC_{arom,meta}$); 134.1 ($C_{arom,quat}$); 156.9 (q, *J* = 32.2 Hz, C=N). **^{19}F NMR** (376 MHz, $CDCl_3$): δ -72.4 (3F, s, CF_3). **IR** (ATR, cm^{-1}): $\nu_{C=N}$ = 1682; ν_{max} = 1198, 1177, 1125, 1084, 731, 704, 694, 642. **MS** (70 eV): m/z (%) 244 ($[M+H]^+$, 100). **GC-MS** (EI): m/z (%) 243 (M^+, 15), 200 (32), 152 (100), 118 (56), 91 (96), 65 (13), 57 (27), 41 (20).

6.4.2 Synthesis of trifluoromethyl amines 26

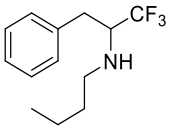
Trifluoromethyl amines **26** were synthesized *via* reduction of the corresponding imines **25** with lithium aluminum hydride. The synthesis of *N*-(1,1,1-trifluoro-3-phenylpropan-2-yl)propan-1-amine **26a** is described as a representative example. The preparation method was again largely taken from the same PhD thesis conducted at SynBioC.[188] To an ice-cooled solution of (*E*)-1,1,1-trifluoro-3-phenyl-*N*-propylpropan-2-imine **25a** (170 mg, 0.74 mmol) in dry diethyl ether (5 mL) was carefully added 0.75 mL of $LiAlH_4$ 1 M solution in THF (0.75 mmol, 1 eq). The ice bath was then removed and the reaction was stirred for 15 minutes at room temperature. The reaction mixture was then cooled to 0 °C with an ice bath and 1 mL of demineralized water was carefully added to quench the remaining $LiAlH_4$. The formed salts were then filtered off over a pad of Celite®. The filter pad was washed with diethyl ether (2 x 10 mL) and the filtrate was washed with brine (20 mL), dried with K_2CO_3, and concentrated *in vacuo* to yield *N*-(1,1,1-trifluoro-3-phenylpropan-2-yl)propan-1-amine **26a** (95%).

***N*-(1,1,1-Trifluoro-3-phenylpropan-2-yl)propan-1-amine 26a**



Colorless oil. Yield: 95%. R_f = 0.30 (hexane/EtOAc 90/10). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 0.71 (3H, t, *J* = 7.4 Hz, CH_3); 1.06 (1H, br s, NH); 1.27 (2H, ~sextet, *J* = 7.2 Hz, $C\underline{H}_2CH_3$); 2.40 (1H, d x t, *J* = 11.3, 7.1 Hz, (HCH)NH); 2.64 (1H, d x d, J_{AB} = 14.0 Hz, *J* = 10.5 Hz, (HCH)CH); 2.64-2.70 (1H, m, (HCH)NH); 3.10 (1H, d x d, J_{AB} = 14.0 Hz, *J* = 3.2 Hz, (HCH)CH); 3.18-3.27 (1H, m, CH); 7.24 (2H, d, *J* = 7.6 Hz, 2 x $CH_{arom,ortho}$); 7.25-7.29 (1H, m, $CH_{arom,para}$); 7-32-7.35 (2H, m, 2 x $CH_{arom,meta}$). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 11.2 (CH_3); 23.1 ($\underline{C}H_2CH_3$); 35.1 (q, *J* = 2.2 Hz, $\underline{C}H_2CH$); 50.8 (CH_2NH); 61.1 (q, *J* = 27.3 Hz, CH); 126.8 (q, *J* = 283.5 Hz, CF_3); 127.0 ($HC_{arom,para}$); 128.7 (2 x $HC_{arom,meta}$); 129.2 (2 x $HC_{arom,ortho}$); 136.7 ($C_{arom,quat}$). **^{19}F NMR** (376 MHz, $CDCl_3$): δ -75.3 (3F, d, *J* = 7.1 Hz, CF_3). **IR** (ATR, cm^{-1}): ν_{max} = 1267, 1142, 1113, 1078, 748, 698. **MS** (70 eV): m/z (%) 232 ($[M+H]^+$, 100). **GC-MS** (EI): m/z (%) 231 (M^+, 2), 202 (13), 140 (100), 109 (12), 98 (29), 91 (23), 43 (18).

***N*-(1,1,1-Trifluoro-3-phenylpropan-2-yl)butan-1-amine 26b**



Colorless oil. Yield: 72%. R_f = 0.48 (hexane/EtOAc 90/10). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 0.77 (3H, t, *J* = 7.2 Hz, CH_3); 1.00 (1H, br s, NH); 1.05-1.19 (2H, m, $C\underline{H}_2CH_3$); 1.20-1.27 (2H, m, $C\underline{H}_2CH_2CH_3$); 2.42 (1H, d x t, *J* = 11.5, 6.8 Hz, (<u>H</u>CH)NH); 2.64 (1H, d x d, J_{AB} = 14.1 Hz, *J* = 10.5 Hz, (<u>H</u>CH)CH); 2.67-2.73 (1H, m, (HC<u>H</u>)NH); 3.10 (1H, d x d, J_{AB} = 14.1 Hz, *J* = 3.3 Hz, (HC<u>H</u>)CH); 3.17-3.26 (1H, m, CH); 7.23 (2H, d, *J* = 7.6 Hz, 2 x $CH_{arom,ortho}$); 7.26-7.29 (1H, m, $CH_{arom,para}$); 7.32-7.35 (2H, m, 2 x $CH_{arom,meta}$). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 13.7 (CH_3); 19.9 ($\underline{C}H_2CH_3$); 32.0 ($\underline{C}H_2CH_2CH_3$); 35.1 (q, *J* = 2.2 Hz, $\underline{C}H_2CH$); 48.6 (CH_2NH); 61.2 (q, *J* = 27.3 Hz, CH); 126.8 (q, *J* = 283.4 Hz, CF_3); 127.0 ($HC_{arom,para}$); 128.7 (2 x $HC_{arom,meta}$); 129.2 (2 x $HC_{arom,ortho}$); 136.7 ($C_{arom,quat}$). **^{19}F NMR** (376 MHz, $CDCl_3$): δ -75.3 (3F, d, *J* = 7.0 Hz, CF_3). **IR** (ATR, cm^{-1}): ν_{max} = 1261, 1142, 1111, 1078, 748, 696. **MS** (70 eV): m/z (%) 246 ($[M+H]^+$, 100). **GC-MS** (EI): m/z (%) 245 (M^+, 3), 202 (23), 154 (100), 112 (81), 109 (18), 98 (21), 91 (35), 57 (19), 41 (15).

6.4.3 Photometric characterization of the IREDs

6.4.3.1 Protein concentration

The Pierce BCA™ Protein Assay was used to analyze the protein concentrations of the enzyme solutions. This assay is based on the reduction of Cu^{2+} to Cu^+ by proteins at basic pH. The formed Cu^+ ions are subsequently chelated with two bicinchoninic acid (BCA) molecules, resulting in an intensely colored purple complex. This complex can be quantitatively measured at 562 nm on a plate reader. The protein concentration is calculated *via* a calibration curve of bovine serum albumin (BSA) (concentrations 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg mL^{-1}). Each enzyme sample was diluted with a factor 10, 100 and 1000 and for every dilution, the average of three measurements was used.

6.4.3.2 Enzyme activity

The enzyme activity of the IREDs toward 2-methyl-1-pyrroline **28** and trifluoromethyl imine **25a** was determined by measuring the consumption of NADPH in a 96 well plate at 340 nm. Reactions were started by adding the enzymes to the working solutions and were monitored for 10 minutes with measurements made every 20 seconds. A calibration curve of NADPH (concentrations 750, 500, 250, 100, 75, 50, 25 and 0 µM) was freshly made for each experiment to convert the absorbance measurements into concentrations.

6.4.4 IRED-catalyzed reduction of trifluoromethyl imine 25a with cofactor regeneration

Reaction solutions were prepared to obtain final concentrations of 10 mM imine **25a** (250 mM stock in DMF), 4% (*v*/*v*) DMF, 0.93 mM NADPH, 20 mM glucose-6-phosphate, 5 U mL^{-1} glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides* from Sigma Aldrich) and 50 mM sodium

phosphate buffer (pH 7.0). The final volume of the reactions was 1100 µL. Imine **25a** was added last in order to minimize hydrolysis before the start of the enzymatic reduction. The reactions were started by the addition of IRED (10 mg mL^{-1} stock) to the reaction solutions. Reactions were incubated at 25 °C with shaking (250 rpm). The sampling procedure is as follows: a 500 µL sample is taken and transferred to a glass vial with 30 µL of 10 M NaOH for inactivation of the enzyme and basification of the solution. The sample is subsequently extracted twice with 500 µL dichloromethane (CH_2Cl_2), each time waiting about 10 min for the layers to partially separate and carefully collecting the bottom organic phase with a needle and syringe. Lastly, the sample is dried with magnesium sulfate ($MgSO_4$) and filtered through a syringe filter. Due to rapid evaporation of the solvent, samples are replenished with fresh, dry dichloromethane to a final volume of about 1 mL (relative concentrations are not affected). Samples were then loaded onto the GC-MS instrument for analysis.

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