

***Application of biocatalysts in the resolution
of peptidomimetic products
from multi-component reactions***

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Declaration

I declare that the work presented in this dissertation was carried out exclusively by myself under the supervision of Dr. M.L. Bode and Professor D. Brady. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

A handwritten signature in black ink, appearing to be 'Fatima Kola', written above a horizontal line.

Fatima Kola

May 2017

Abstract

A library of potentially bioactive peptidomimetic compounds has been created using the Ugi and Passerini multi-component reactions. The products were isolated as racemic mixtures which we aimed to separate by enzymatic means.

The Ugi enantiomers were obtained in exceedingly low yields due to considerable byproduct formation encountered with the use of ammonia as reactant and methanol as solvent. A threefold increase in the yields was achieved when employing the less nucleophilic 2,2,2-trifluoroethanol as solvent. An endeavour to resolve the racemates using proteases and penicillin G acylase proved unsuccessful, possibly attributable to the poor solubility of the Ugi products in a broad range of organic solvents.

Enantiomers obtained from the Passerini reaction were efficaciously hydrolysed by lipase enzymes. Hence, the enantioselectivity of the hydrolysis reactions was explored by the employment of chiral HPLC and Mosher ester analysis. The biocatalysts displayed adequate selectivity for one of the adducts, making prospective separation of the racemic mixture viable.

An investigation into the diastereoselectivity of the Passerini reaction was also performed. The application of phenylalanine-derived chiral starting materials in the reaction was seen to have a slight effect on reaction diastereoselectivity, giving on average a 2:1 diastereomeric ratio. Slightly greater d.r. values were obtained when bulkier isocyanides were employed. The diastereomeric mixtures were effectively separated via preparative HPLC to obtain pure compounds. ^{13}C NMR spectroscopy indicated a trend in the chemical shift values for the newly formed stereogenic carbon centres for most of the two separated diastereomers. The C-2 values of most of the minor diastereomers appeared more downfield in comparison to the major diastereomers. Distinguishing the stereochemistry of the major and minor diastereomers through X-ray crystallography was not possible as the compounds were non-crystalline. Thus the configurations of the newly formed stereogenic centres of the major compounds were assigned by analogy with similar compounds previously identified in our laboratory. The major compounds were described as *R* at the newly formed stereogenic centre when L-phenylalaninal was used, and the minor products *S*. When D-phenylalaninal was used, the major diastereomer was assigned an *S* configuration at the newly formed stereocentre and the minor one was assigned *R*. The formation of the major diastereomer could be explained using a Felkin-Anh chelation controlled model, which has been observed in other reactions of mono-protected amino aldehydes.

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
Boc	<i>tert</i> -butoxycarbonyl
(Boc) ₂ O	Boc-anhydride
CCl ₄	carbon tetrachloride
CDCl ₃	deuterated chloroform
CDI	1,1'-carbonyldiimidazole
CHCl ₃	chloroform
CH ₂ Cl ₂	dichloromethane
CSIR	Council of Scientific and Industrial Research
d	day (s)
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
d.r.	diastereomeric ratio
e.e.	enantiomeric excess
e.r.	enantiomeric ratio
Et ₃ N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
FDA	Food and Drug Administration
h	hour (s)
HCl	hydrochloric acid
HIV	Human Immunodeficiency Virus

H ₂ O	water
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
H ₂ SO ₄	sulfuric acid
IPA	propan-2-ol
IR	infrared
KHSO ₄	potassium bisulfate
KOH	potassium hydroxide
LiAlH ₄	lithium aluminium hydride
MBE	2-methyl-but-3-ene-2-ol
MeCN	acetonitrile
MeOH	methanol
MgSO ₄	magnesium sulphate
min	minute (s)
m.p.	melting point
N ₂	nitrogen
Na ₂ CO ₃	sodium carbonate
NaOH	sodium hydroxide
NMR	Nuclear Magnetic Resonance
POCl ₃	phosphoryl chloride
PPh ₃	triphenylphosphine
R _f	retention factor
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	Thin Layer Chromatography

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Chapter 1: Introduction

1.1 Peptidomimetic Compounds

Proteins and peptides are bioactive molecules that are central to a multitude of physiological processes. They fulfil both structural roles - strengthening and preserving cells and tissues, and functional roles.¹⁻³ As enzymes, hormones and neurotransmitters, peptides can catalyse reactions, regulate gene expression and participate in cell signalling and transduction. Peptides are also involved in the advancement of several diseases, hence scientists have gained interest in developing these molecules as therapeutic agents against HIV, cancer and a range of other diseases.^{4, 5} However, the potential of these biomolecules as drug candidates is severely limited by their unfavourable pharmacological properties. Peptides and proteins are ordinarily poorly absorbed, rapidly degraded and exhibit low specificity which leads to undesirable side effects.⁶⁻⁸ Subsequently, peptidomimetic compounds have been synthesised to overcome these drawbacks.

A peptidomimetic compound is an organic molecule that resembles the structure and biological activity of a natural peptide or protein, but has superior properties that lead to its function as a drug.⁹⁻

¹¹ This is achieved by structurally altering existing peptides, predominantly through incorporating unnatural amino acids into their backbone and by modification of side chains which results in enhanced stability against protease degradation, improved specificity and bioavailability and a considerable increase in activity. This holds peptidomimetics as a valuable tool for the progression of novel anti-viral, anti-malarial, anti-tumour and immunosuppressant drugs.^{2, 12}

Lopinavir, ritonavir and saquinavir (Figure 1) are some well-known examples of successfully synthesised peptidomimetic drugs that are FDA-approved as HIV protease inhibitors. HIV protease inhibitors (PIs) are compounds that inhibit the function of the HIV protease enzyme, making it an effective treatment for HIV/AIDS sufferers.¹³ These drugs structurally mimic the natural substrate of the virus' enzyme but contain a non-scissile hydroxyethylamine moiety in place of the normal amide group of the viral peptide (Figure 1). These compounds inhibit the enzyme's ability to cleave polypeptides into functional proteins which is crucial for replication of the virus, rendering HIV inactive.¹³⁻¹⁷

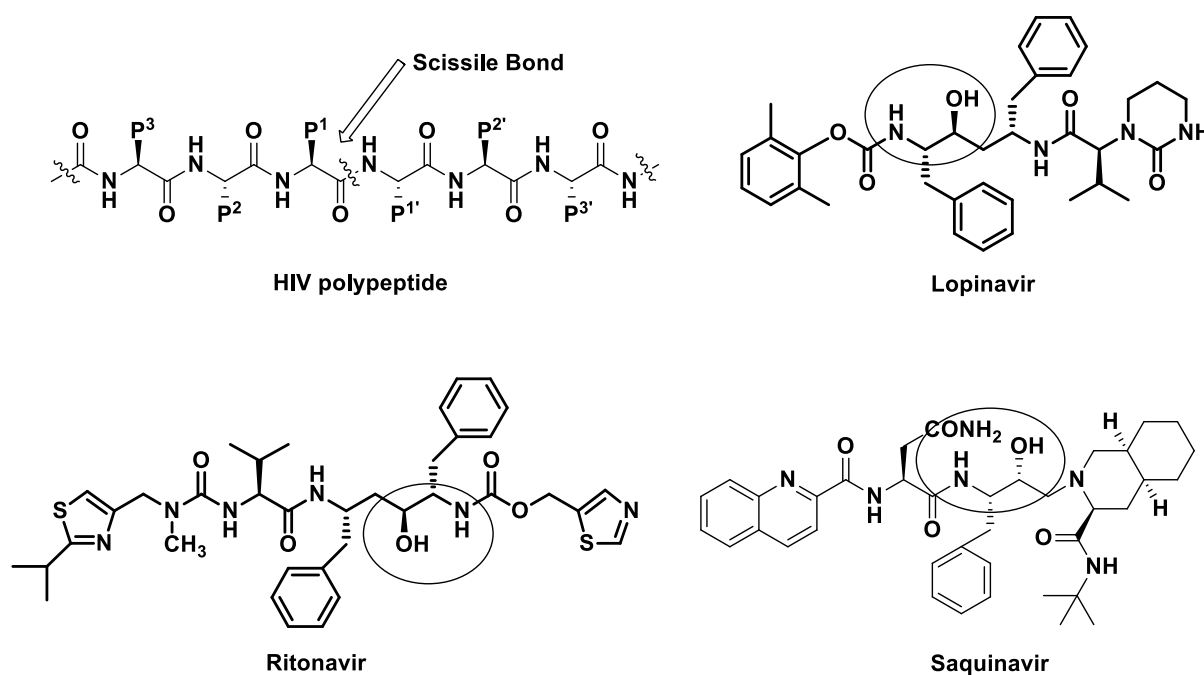


Figure 1: Scissile bond of the viral polypeptide versus the non-cleavable hydroxyethylamine core of the HIV PIs.¹⁸

Another renowned class of peptidomimetics are angiotensin-converting enzyme (ACE) inhibitors which are used to treat hypertension. ACE is a metalloprotease that operates analogously to HIV protease and is responsible for the cleavage of the peptide angiotensin I to angiotensin II which both directly and indirectly increase blood pressure.¹⁹ Thus ACE inhibitors like captopril and enalapril disturb this cascade of reactions, normalising blood pressure with enhanced bioavailability and less side-effects than non-peptidic antihypertensive drugs. Additionally these inhibitors are also active diabetic nephropathy and congestive heart failure medications.²⁰⁻²³ The structure of captopril was based on a nanopeptide, teprotide (Figure 2) contained in the venom of the poisonous snake *Bothrops jararaca*. This peptide is a powerful inhibitor of ACE and results in an instantaneous decrease in blood pressure, making it a fascinating tactic for the snake to capture its prey. Although the activity of teprotide was undeniable, it was costly and not orally bioavailable. Scientists deduced that the proline residue at the terminus was responsible for the activity of this peptide, hence after considerable modification they designed captopril which retained the vital amino acid and included a sulfhydryl functionality which led to an astounding increase in activity of a thousand-fold.^{24, 25}

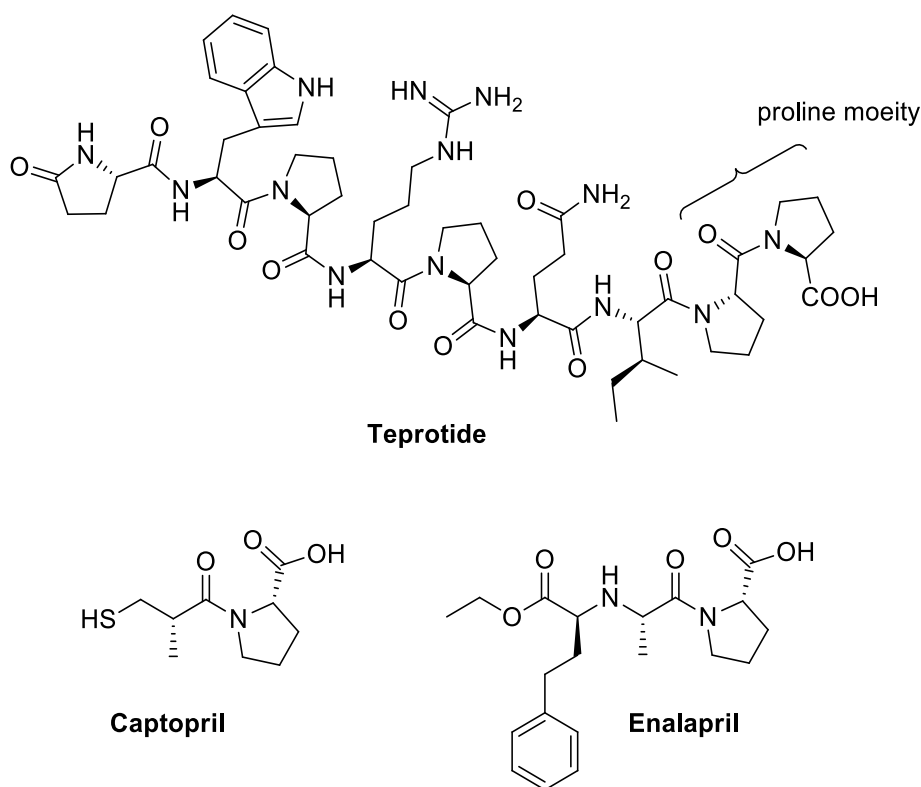
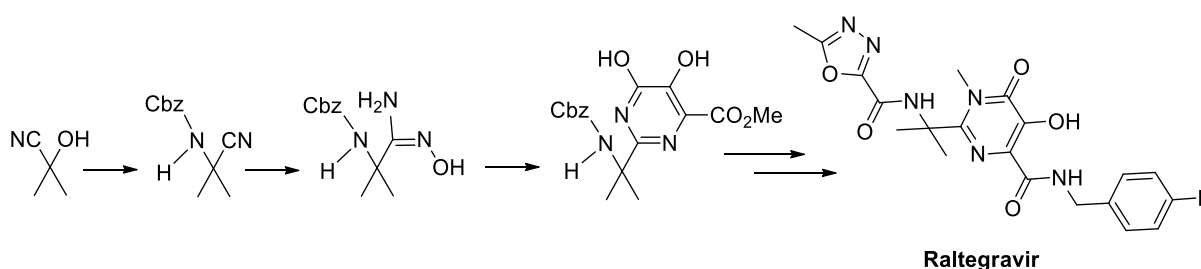


Figure 2: Peptidomimetic ACE inhibitors.

The initial strategy for synthesising peptide mimics entails identification of essential amino acid residues mandatory for bioactivity through structure/conformation/activity relationships of the native peptide.^{26, 27} Scaffolds are then designed to modify the peptide by introducing non-hydrolysable amide bond isosteres or conformational restraints until a hit compound is found. Syntheses of these analogues typically involves a series of time-consuming linear reactions and is often not simple to achieve libraries of structurally diverse compounds.²⁷⁻³⁰ For instance Merck designed an anti-HIV drug Raltegravir (Scheme 1), which contains a 1,3,4-oxadiazole five-membered heterocyclic ring.³¹ The synthetic route is lengthy, comprising of six linear reactions with protection and deprotection steps. The complete synthesis requires over 100 hours of reaction time excluding chromatographic purification.³¹ Hence, the development of more straightforward reactions that can be used in a “model synthesis” is imperative.



Scheme 1: Synthesis of Raltegravir.

1.2 Multicomponent Reactions

A “model chemical synthesis” is one in which the anticipated product is attained in close to a 100% yield from a reaction comprising the minimal number of steps. The experimental procedure should be safe, effortless and fast whilst utilising inexpensive materials and techniques which pose no harm to the environment.^{32, 33}

Multi-component reactions (MCRs) first established by Laurent and Gerhardt in 1838 closely reflect a “model chemical synthesis”.³² MCRs are one-pot reactions in which three or more reactants combine to form a single composite product.^{34, 35} The reaction occurs in a series of fundamental steps which proceeds to the final irreversible reaction where the compound is obtained.³⁶ Since a variety of starting materials with diverse functional groups can be incorporated, a vast library of compounds can be created to be screened for use as possible therapeutic agents.³⁷⁻³⁹

MCRs are atom-economical in that most of the reactant material is included in the product (Figure 3). The amount of solvent used is also lessened as the process is completed in one step, deeming it efficient and environmentally-friendly. The formation of by-products is negligible which results in a higher yield as compared to a multistep synthesis. Perhaps the most advantageous feature of MCRs is post-transformation and modification of the products which leads to increasingly structurally complex compounds with potential bioactivity.^{34, 40}

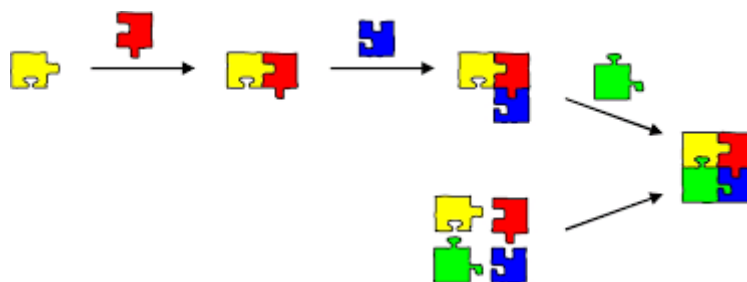
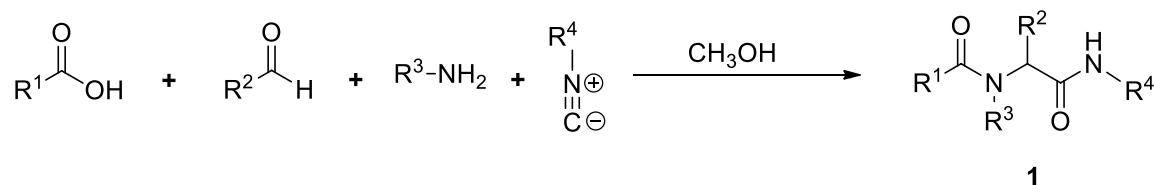


Figure 3: Illustration of a stepwise (multistep) reaction in comparison to a multicomponent reaction (MCR) which occurs in a single step.⁴¹

1.2.1 Ugi Reaction

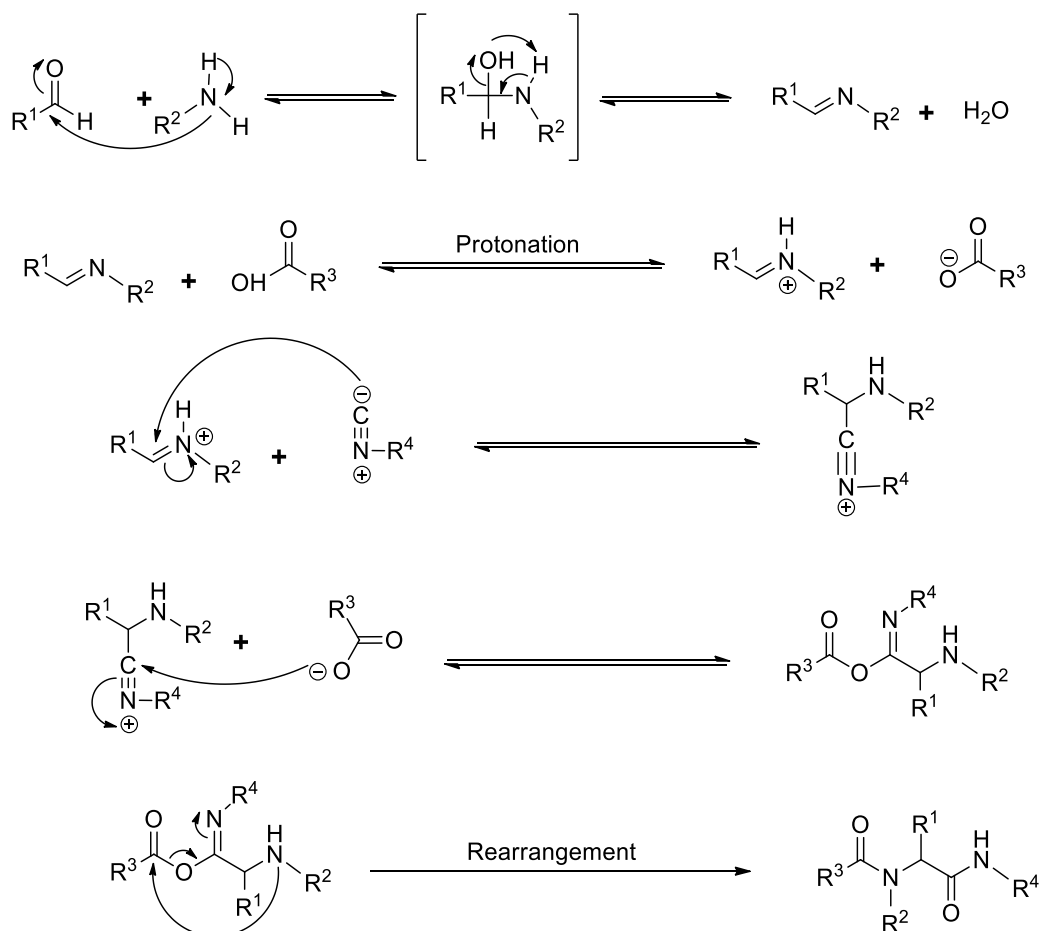
The Ugi reaction accomplished by Ugi *et al.* in 1959 is an example of an isocyanide model of a MCR that is often used in medicinal and heterocyclic chemistry.⁴² Amongst its variations is the Ugi-four component reaction (U-4CR) which includes condensation of a carboxylic acid, aldehyde (or ketone), primary or secondary amine and an isocyanide to generate a stereogenic centre in the α -acylaminocarboxamide product **1** (Scheme 2).⁴³



Scheme 2: Ugi-four component reaction in methanol.

The U-4CR mechanism depends on the resonance of the isocyanide which converts from the C^2 to the stable C^4 form, allowing the C to uniquely function as both an electrophile and a nucleophile.³² The reaction is usually performed at room temperature in polar, aprotic solvents; but success with methanol (and trifluoroethanol) as well as in aqueous media was reported by Thompson *et al.* and Mironov *et al.*, respectively.^{40, 44} Scheme 3 outlines the U-4CR mechanism in which the carbonyl compound (aldehyde or ketone) and the amine initially precondense to form the Schiff base (imine) with the release of water. The carboxylic acid then protonates the imine nitrogen in the next step. The

third step involves the isocyanide reacting with the electrophilic iminium cation to give the nitrilium intermediate, which in turn reacts with the nucleophilic acid anion. The resulting intermediate then irreversibly undergoes intramolecular rearrangement to generate the final Ugi product.³²



Scheme 3: Mechanism of the U-4CR.

Mironov *et al.* aimed to achieve a procedure for compound library production that is both easy and eco-friendly.⁴⁴ Taking into account that high-throughput screening of biomolecules is conducted in water at physiological pH and room temperature, they prepared organic compounds via the U-4CR in an aqueous medium rather than using regular organic solvents. Although water is considered a poor solvent for the majority of organic molecules, its extraordinary properties can lead to advantageous results in synthetic chemistry. For instance, organic compounds aggregate in certain regions in water

owing to their hydrophobic nature. These regions are greatly concentrated and thus collisions between particles are quicker, resulting in faster addition reactions. Also aggregation of organic particles occurs in particular orientations which results in notable product selectivity.^{44, 45}

Using levulinic acid with several amines and isocyanides, Mironov *et al.* demonstrated that the Ugi reaction is successfully performed in aqueous conditions in good yields provided that the concentration of reactants are in the high range of 0.5 – 2.0 M.⁴⁴ For starting materials that exhibited poor solubility in water, supersaturated solutions and surfactants were used and equally good results were obtained. Hence the feat of the Ugi reaction depends not only on the type of solvent used but also on the concentration of starting material present.⁴⁴

To obtain a collection of 5-aminothiazole and oxazole derivatives, Thompson and Chen executed the U-4CR with ammonia as the primary amine constituent.⁴⁰ Exceptionally low yields were obtained in methanol solvent due to a large number of byproducts gained from side reactions often experienced when using ammonia in the Ugi reaction. This is due to the involvement of the alcoholic solvent in the presence of excess aldehyde via a six-component coupling (6-CC) reaction which leads to the formation of the most predominant byproduct **2** (Figure 4). In earlier research, Kazmaier *et al.* realised that since **2** was obtained using methanol, a less nucleophilic solvent like 2,2,2-trifluoroethanol would prevent possible side reactions thus leading to increased yields.^{40, 46, 47} Thompson and Chen proved this to be true and achieved nearly a twofold increase in the yields using 2,2,2-trifluoroethanol in comparison to methanol.⁴⁰ In addition the protocol was simplified as fewer purification steps were required since generation of byproducts was almost negligible.

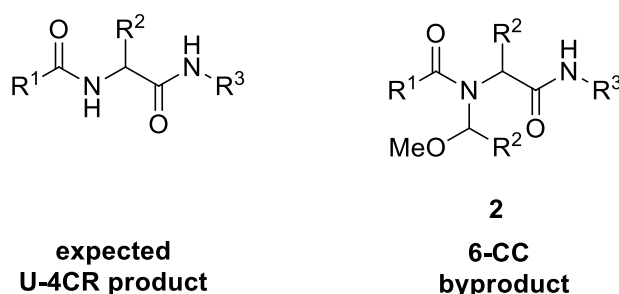
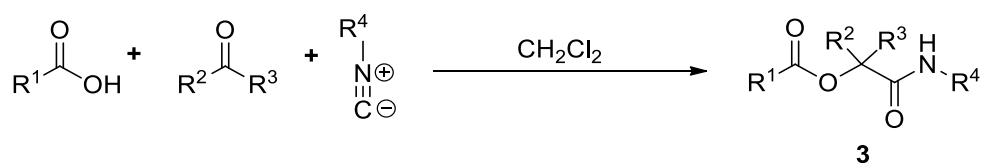


Figure 4: Major byproduct **2** obtained with the U-4CR using ammonia and methanol.

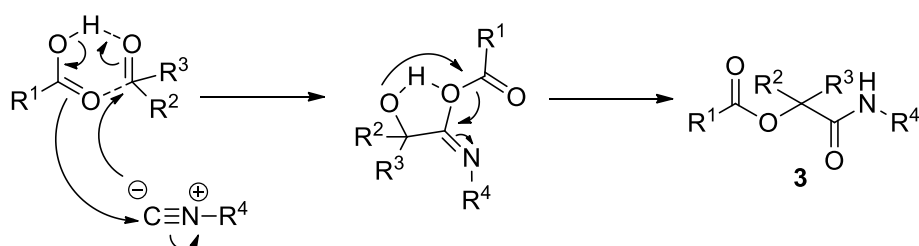
1.2.2 Passerini Reaction

The Passerini reaction is another isocyanide-type MCR akin to the U-4CR but features only three components, namely an acid, aldehyde (or ketone) and isocyanide which forms α -hydroxycarboxamide compounds **3** (Scheme 4). To date there has been extensive use of both these MCRs in drug design and production of substantial compound libraries.⁴⁸⁻⁵¹



Scheme 4: Passerini reaction performed in dichloromethane.

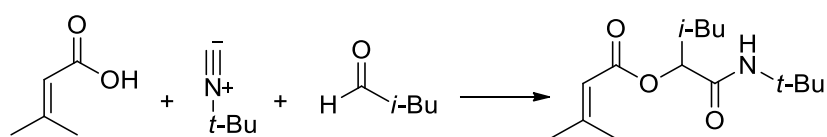
The Passerini reaction can be carried out in polar solvents, but was originally believed to occur more rapidly in aprotic solvents such as CH_2Cl_2 (Scheme 4). The latter route may follow a concerted non-ionic pathway (Scheme 5) which involves H-bonding between the aldehyde and carboxylic acid. The isocyanide carbon acts as a nucleophile and electrophile to form a non-isolatable 5-membered transition state ring. An intramolecular acyl transfer to the adjacent hydroxyl group leads to rearrangement to the stable Passerini product **3**.⁵²



Scheme 5: Mechanism of the Passerini reaction in aprotic solvents.

Just as with the U-4CR, Pirrung *et al.* also proved the effectiveness of conducting the Passerini reaction in aqueous media.⁵³ The reaction demonstrated in Scheme 6 was performed in both dichloromethane

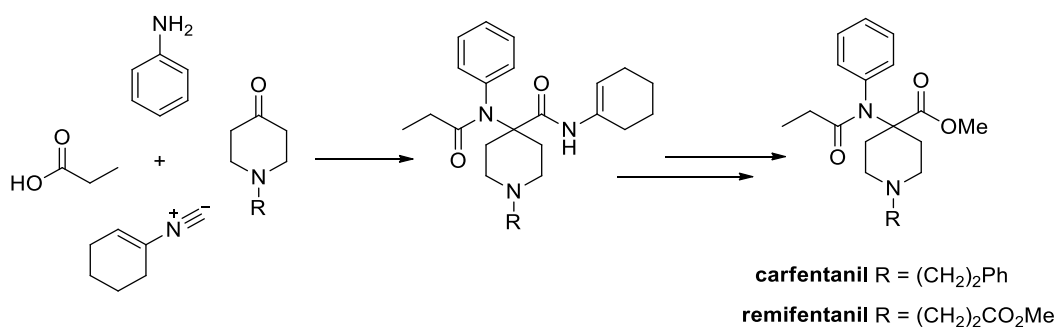
and water. In contrast to previous reports, Pirrung found that the rate of the reaction was accelerated 18-fold in water with 100% conversion achieved in only 3.5 hours.⁵³ Dichloromethane afforded a 45% yield of the product after 18 hours as opposed to the excellent yield of 95% obtained in aqueous media at room temperature. The experiment was also carried out in water at 4 and 50 °C and outstanding yields were likewise noted as compared to organic solvents. Recent reports have credited the superior reaction rates of this MCR in water to the improved H-bonding in the transition state (Scheme 5) as well as the hydrophobic effect. This finding not only provides an efficient method for the Passerini reaction, but also presents an eco-friendly approach to complex organic molecules.⁵³



Scheme 6: Passerini reaction performed in dichloromethane and water.

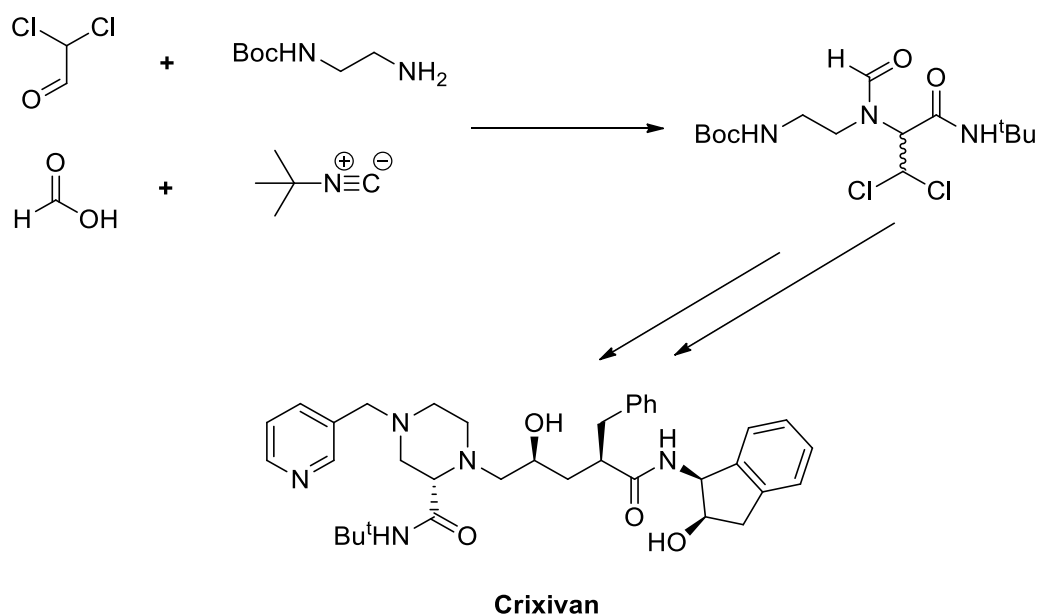
1.2.3 Application of MCRs

Synthesis of the substructure 4-aminopiperidine-4-carboxylic acid present in carfentanil and remifentanil (Scheme 7) - opioid analgesics used for large animals and humans, respectively, typically comprises of 8 steps.⁵⁴ Malaquin and co-workers attempted a simpler approach to this preparation using the Strecker multicomponent reaction but obtained the desired compound in very poor yields.³⁷ They later demonstrated that the Ugi reaction was more synthetically efficient and yielded no side-products owing to the stable α -acylaminocarboxamide product formed (Scheme 7).³⁷



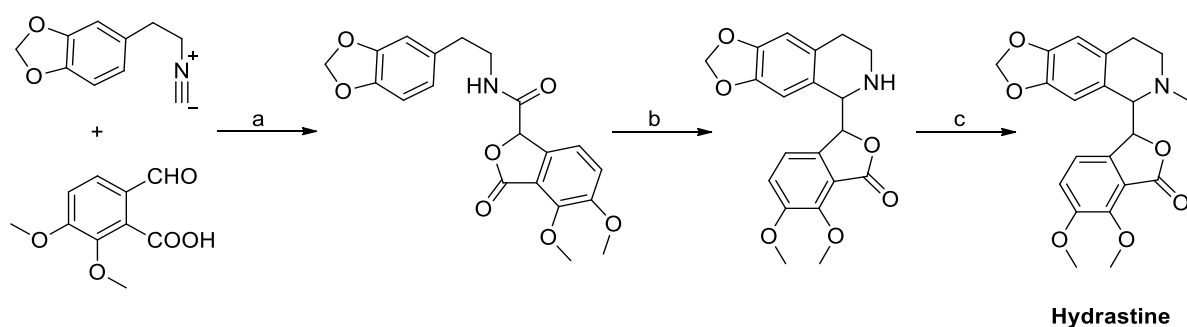
Scheme 7: Preparation of 4-aminopiperidine-4-carboxylic acid derivatives.

Merck has similarly employed the U-4CR (with subsequent enantioselective hydrogenation) in the manufacture of indinavir (CrixivanTM), another potent HIV protease inhibitor approved by the FDA in 1996 (Scheme 8). Indinavir is a peptidomimetic compound which would conventionally be prepared in a 20-step synthesis, hence introduction of the U-4CR resulted in faster production, ultimately leading to a more affordable consumer product.^{32, 55}



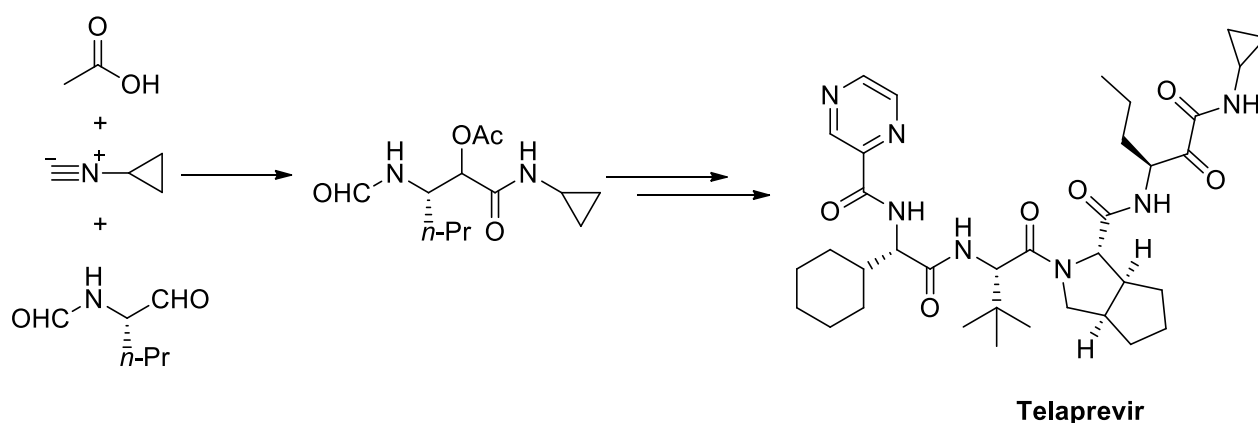
Scheme 8: Synthesis of CrixivanTM, an approved HIV protease inhibitor using the U-4CR.

The Passerini reaction has also been utilised in many instances. One notable application is the preparation of a phthalideisoquinoline alkaloid, hydrastine, which naturally occurs in the *Hydrastis canadensis* plant species, more commonly known as goldenseal (Scheme 9).⁵⁶ This alkaloid has several pharmacological properties which include treatment of upper respiratory tract infections and postpartum haemorrhage.⁵⁷ A four-step complete synthesis of hydrastine was described in 1981 by Falck *et al.* of which an intramolecular Passerini reaction was the pivotal step.⁵⁸ The preparation started with an alkylation reaction which yielded 3,4-methylenedioxyphenethylisocyanide. This in turn reacted with opianic acid that functions as both the acid and aldehyde component of the MCR, followed by ring-closure, hydrogenation and reductive amination to form the final compound.⁵⁸



Scheme 9: Synthesis of hydrastine. Reagents: a) CH_2Cl_2 b) POCl_3 , MeCN, PtO_2 , H_2 c) NaBH_3CN , COH_2 .

The Hepatitis C virus (HCV) impacts more than 200 million people globally. It has become a major health concern as it can lead to cirrhosis, liver failure and cancer. Presently there are no vaccines for this infection, but treatment comprises of HCV NS3 protease inhibitors. The Passerini reaction is involved in the complete synthesis of the HCV PI telaprevir (Scheme 10), a large molecule with complex stereochemistry. The reaction gives rise to diastereomers by coupling of a chiral aldehyde, cyclopropyl isocyanide and acetic acid with a favourable d.r. of 78:22.^{59, 60}



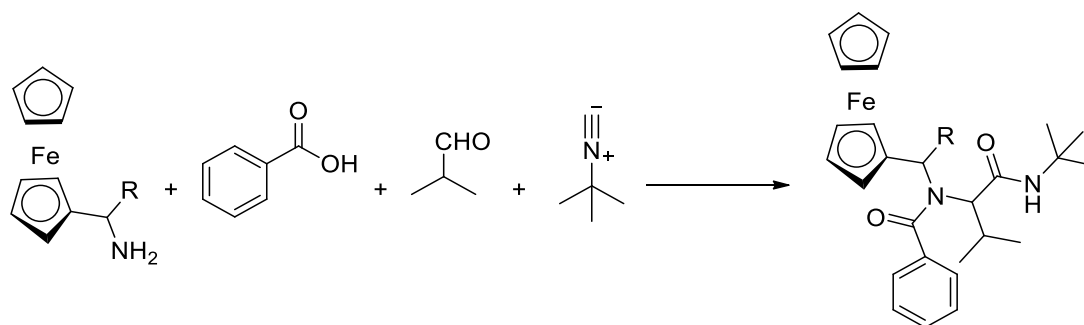
Scheme 10: Diastereomeric Passerini reaction as part of the total synthesis of telaprevir.

1.2.4 Diastereoselectivity of MCRs

When starting with achiral reactants these isocyanide-MCRs lead to a 50:50 mixture of enantiomers as a new stereogenic centre is formed during the course of the reaction. If one of the starting materials already contains a stereogenic centre, a mixture of diastereomers will then be obtained. As a result enantiomerically/diastereomerically pure compounds are not achieved. This has a negative consequence, especially in drug design where only one enantiomer will exhibit activity and the other may be inactive or even harmful. However numerous post-modification processes, including enzymatic resolution which will be discussed later, can be applied to the products to overcome this matter.⁶¹

Various studies have been done on the diastereoselectivity of both the Ugi and Passerini reactions. Chiral isocyanides and carboxylic acids have been found to have no momentous effect on the selectivity of the U-4CR, affording mostly 1:1 ratios of diastereomeric mixtures. Chiral aldehydes, especially α -alkyl-containing compounds, exhibit low configurational stability during the Ugi reaction and racemize relatively easily. On the other hand, amines containing a fixed stereogenic centre offer the greatest selectivity in comparison to the other starting materials of the U-4CR, but in most cases the d.r. values are still not exceptional.⁶²

In the early 1970s Ugi *et al.* first described the employment of chiral (+)- α -ferrocenylalkylamines in diastereomeric U-4CRs with benzoic acid, *iso*-butyraldehyde and *t*-butylisocyanide.^{63, 64} Changes in the diastereoselectivity were seen under various solvent and temperature conditions, as well as with the alkyl substituent of the amine. When R = methyl (Scheme 11), a fair d.r. of 79:21 was observed in a yield of 90% at 0 °C in a methanolic solvent. Later studies reported an outstanding d.r. of 99:1 when using bulkier ferrocenylamines (R = isopropyl) at -78 °C. Regardless of the great selectivity, the use of these chiral amines have not been further examined due to instability and low recovery of these compounds.^{63, 64}



Scheme 11: Diastereoselective Ugi reaction with chiral ferrocenylalkylamines.

Mandai *et al.* investigated the diastereoselectivity of the U-4CR with α -amino acids and chiral DMAP-derived aldehydes in methanol.⁶⁵ Two aldehydes were utilised, 4-(dimethylamino)-2-pyridinecarboxaldehyde and 4-(dimethylamino)-3-pyridinecarboxaldehyde (Figure 5) which delivered 2- and 3-substituted DMAP analogues, respectively. Using L-valine at an optimal concentration of 0.5 M of the former aldehyde, they discovered that the diastereoselectivity was moderate at 63:37. The structure of the amino acids presumably had no effect on the selectivity of the reaction. Using 4-(dimethylamino)-3-pyridinecarboxaldehyde with L-valine afforded a superior d.r. of 84:16 under identical conditions. The best result of 93:7 d.r. was achieved with L-isoleucine, exhibiting a relationship of the α -amino acid structure and the latter aldehyde. Mandai *et al.* postulated that the preference of the formation of one diastereomer over the other was due to the prevention of steric hindrance from the bulky dimethylamino moiety from the aldehyde and the amino acid side chain during imine formation during the reaction. In addition, X-ray crystallography depicted an *R*-configuration at the new stereogenic centre for the major Ugi diastereomer.⁶⁵

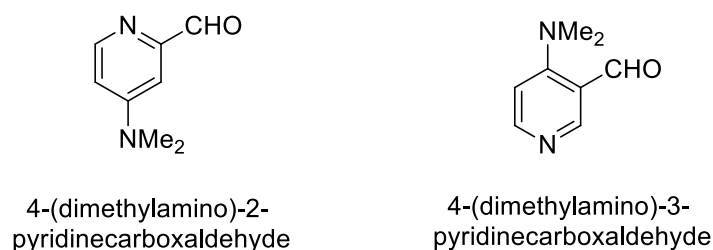
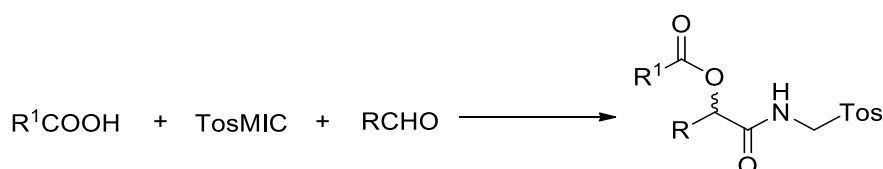


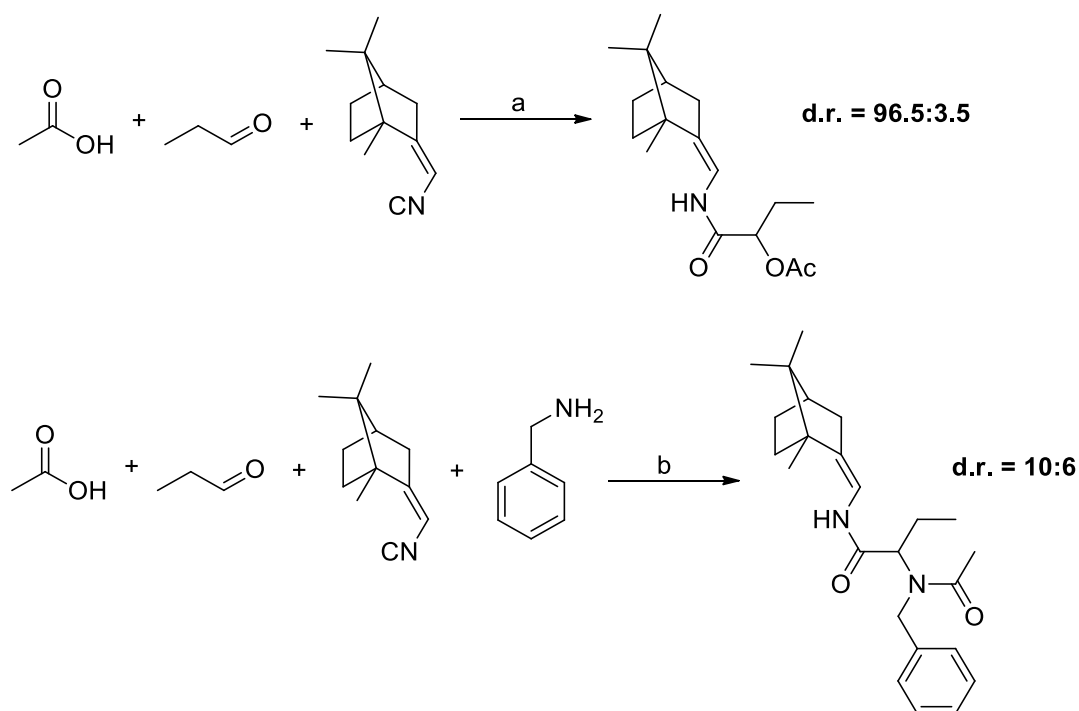
Figure 5: DMAP-based aldehydes.

Conventionally the carbonyl component in the Passerini reaction has only a minor influence on the stereoselectivity, yielding diastereomeric ratios varying between 1:1 and 4:1.⁶⁶ On the contrary, Krishna and co-workers utilised *p*-toluenesulfonylmethyl isocyanide (TosMIC) in innovative asymmetric Passerini reactions with chiral glucose-derived aldehydes and attained average to notable d.e. values of 30 – 90% (Scheme 12).⁶⁷ Although the diastereomers were not separately isolated by chromatographic purification, the d.e.'s were obtained using the integral ratios of the diastereomeric protons from ¹H NMR data. In addition, the absolute configuration at the newly formed stereogenic centre of the major diastereomer was assigned as the '*R*- isomer'. This was also established via coupling constants from ¹H NMR spectroscopy.⁶⁷



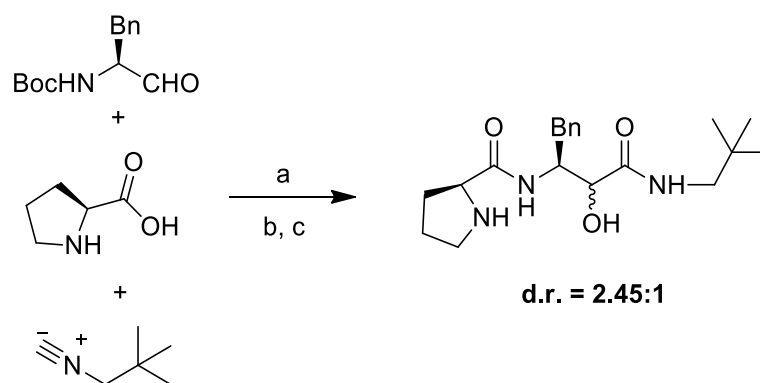
Scheme 12: Diastereomeric Passerini reaction with TosMIC.

In the case of using chiral isocyanides in the Passerini reaction, Bock *et al.* achieved excellent diastereoselectivity using camphor-derived α,β -unsaturated isocyanides with aliphatic aldehydes (Scheme 13). However chiral isocyanides have a negligible effect on the diastereoselectivity of the U-4CR. The Ugi reaction was carried out by the same research group with an additional benzylamine component and the d.r. was remarkably low at 10:6.^{66, 68}



Scheme 13: Diastereoselective MCRs using camphor-derived isocyanides. a) Passerini reaction b) U-4CR.

Banfi *et al.* studied the diastereoselectivity of peptidomimetic compounds as protease inhibitors via the PADAM (Passerini-amine deprotection-acyl migration) approach.⁶⁹ This entailed the primary Passerini product spontaneously undergoing acyl migration once the amine group is deprotected by mediation of TFA. This gives rise to a product consisting of a hydroxyethylene amine entity, which is characteristic of most current PIs. They employed various chiral aldehydes based on amino acids in combination with an assortment of achiral isocyanides and chiral and achiral acids. The d.r. values obtained averaged at 2:1 with a greater value noted for the reaction in Scheme 14. The selectivity remained consistent regardless of the reagents involved. They did not however separate the mixture to ascertain the configuration of the major and minor diastereomers.⁶⁹



Scheme 14: Reagents: a) CH_2Cl_2 b) TFA, CH_2Cl_2 c) Et_3N .

1.3 Enzymatic Kinetic Resolution/ Biocatalysis

Enzymes are biological macromolecules which mediate most chemical transformations in living organisms. They are biocatalysts that decrease the activation energy required for processes, thereby speeding up reactions by up to 10^{21} times that of an uncatalysed reaction.⁷⁰ Catalysis is achieved at a particular site on the enzyme called the active site, at which the enzyme interacts with the reactant or substrate by weak ionic, hydrogen or van der Waals forces. Enzymes are highly specific and only react with substrates that can bind in the active site. The major advantage of this is that all the substrate is converted to the desired product, eliminating the generation of by-products by side-reactions, minimising waste and the negative impact on the environment.⁷¹

Enzymes are proteins which require conditions close to those encountered *in vivo* to function. This generally includes low temperatures between 35 – 40 °C, pH of roughly 7.5 and dilute aqueous solvents. Extreme temperatures and pressures normally result in denaturation of the enzyme, inactivating it. The criteria for enzyme-function is mild, eco-friendly and requires less energy and high-priced machinery, which is the key reason as to why even industries are attracted to biocatalysis for food and cosmetic production. Like all catalysts, enzymes are not altered or consumed during the reaction and can be used repeatedly after each catalytic cycle.⁷²

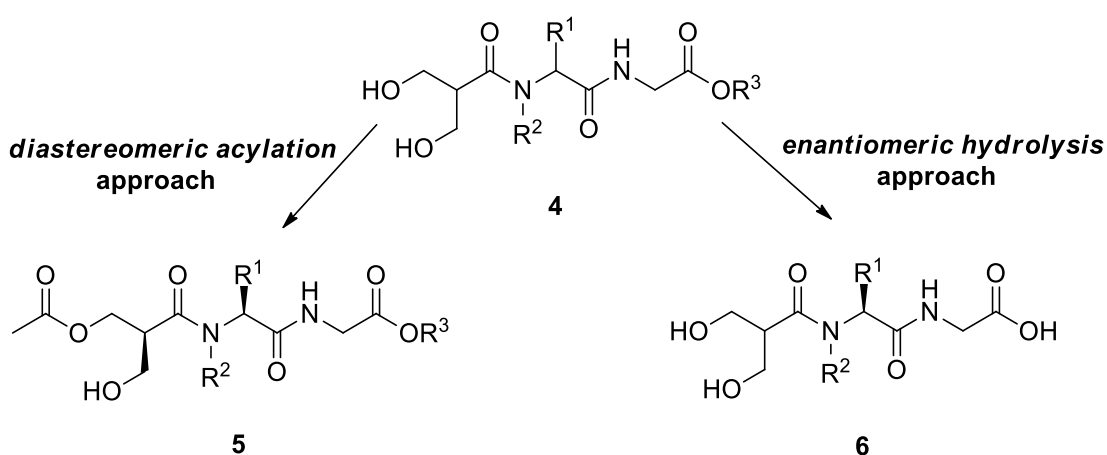
Biocatalysts exhibit chemo-, regio-, and stereo-selectivity - which makes them ideal in resolving racemic mixtures to obtain enantiomerically pure compounds, including those obtained from MCRs.⁷¹

This practice is referred to as enzymatic kinetic resolution (EKR), and occurs because enzymes react specifically or more rapidly with one enantiomer transforming it to the relevant product, and leaves the other unchanged, thereby allowing separation of the mixture.^{61, 73, 74}



Figure 6: Outline of the combination of MCRs and biocatalysis to achieve stereochemically pure compounds.

One illustration of biocatalysis in combination with the Ugi MCR (Figure 6) was reported by Klossowski *et al.*⁷⁵ They set out to synthesise enantiomerically pure 1,3-diol peptidomimetic compounds which were previously used as kinase inhibitors, anticonvulsant agents and can act as intermediates in making effective anticancer treatments. Two EKR approaches were tested- one involved a diastereoselective method using lipase enzymes which acetylated compound **4** to compound **5** (Scheme 15). The other route also employed lipases, and involved an enantioselective approach where the ester moiety of compound **4** was hydrolysed to a carboxylic acid in **6**.⁷⁵



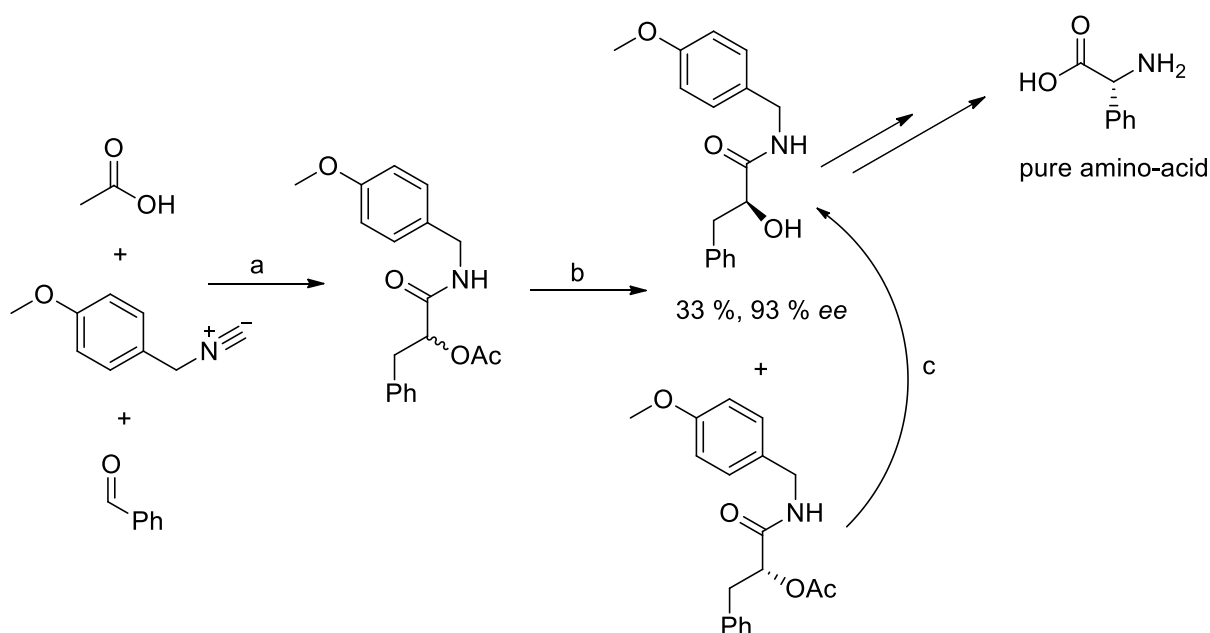
Scheme 15: Enzymatic resolution of 1,3-diol peptidomimetics using lipases.

In the first approach seven different lipases successfully catalysed the acylation reaction. They discovered that the solvent used is imperative in stereoselectivity of the lipase enzyme, and after investigating several organic solvents, diisopropyl ether was used.⁷⁶ Maximum diastereoselectivity was exhibited with lipase *Aspergillus oryzae* with a disappointing d.e. of 48% being obtained.⁷⁵

Six different enzymes were found to hydrolyse the ester group in compound **4** in the second approach. This reaction yielded better results as compared to the acylation method. Exceptional enantioselectivity was observed with *Candida antarctica* and *Rhizopus arrhizus* lipase with e.e. of > 99.9% and 82%, respectively.

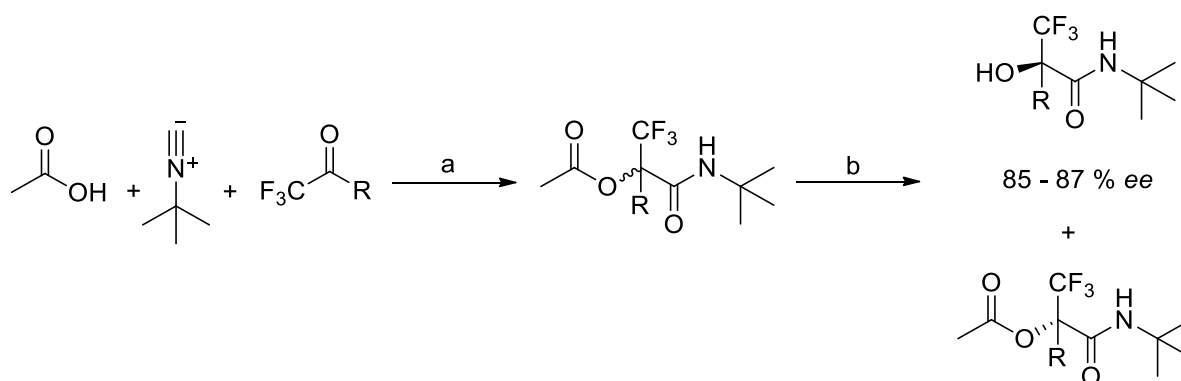
In conclusion Klossowski *et al.* effectively synthesised peptidomimetic compounds using the U-4CR, after which enantiomerically pure products were obtained by EKR with lipase enzymes.⁷⁵ This combination of the U-4CR and biocatalysis proved to be a simple and versatile method in obtaining complex compounds with promising bioactivity.

The same group also employed EKR in conjunction with the Passerini MCR to obtain enantiomerically pure compounds. For the synthesis of α -amino acids, Ostaszewski and colleagues applied the Passerini reaction using phenylacetaldehyde, acetic acid and *p*-methoxybenzyl isocyanide – of which the resultant product was selectively hydrolysed by *Wheat Germ* Lipase in 33% yield with an e.e. of 93% (Scheme 16).^{76, 77} They also ascertained that the hydrophobicity of the solvent greatly influenced the enantioselectivity of the reaction. Later they learnt that addition of a *t*-butyl ester group at the C-terminus and using chloroacetic acid to provide a better leaving group for the hydrolysis, significantly enhanced the selectivity.⁷⁶⁻⁷⁸



Scheme 16: Preparation of α -amino acids using the Passerini reaction with EKR. Reagents: a) CH_2Cl_2 , 20 °C, 48 h b) *Wheat Germ* lipase, 8:2 phosphate buffer pH 7.0/ether, 20 °C, 48 h c) NaOH, MeOH/water, ultrasound, TosCl, Et_3N , CH_2Cl_2 , 20 min.

Scheme 17 shows the Passerini reaction implemented by Kourist *et al.* to prepare α,α -dialkyl- α -hydroxylic acid esters, which are valuable intermediates in drug design.⁷⁹ From more than 40 enzymes that were tested, an esterase of metagenomic origin afforded the best result.^{61, 79}



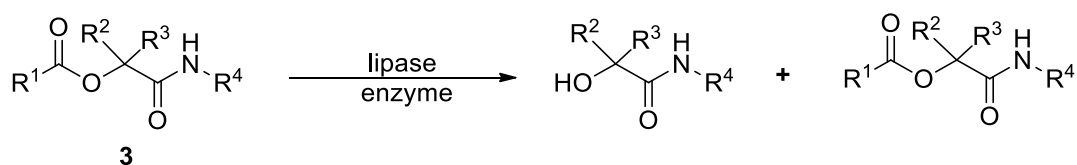
Scheme 17: Synthesis of enantiomerically pure tertiary alcohols employing the Passerini reaction in conjunction with enzymatic hydrolysis. Reagents: a) CH_2Cl_2 b) Esterase, phosphate buffer, DMF.

1.4 Proposed Synthesis

The focus of this project is the synthesis of a library of peptidomimetic compounds using the Ugi and Passerini multicomponent reactions. The racemic mixtures generated from the reactions will be resolved by use of biocatalysts to obtain pure compounds with prospective bioactivity.

First the U-4CR will be employed to prepare enantiomers as illustrated in Scheme 2. To afford Ugi products that are easily accessible to enzymes, we intend to use ammonia as the amine component in the reaction. This not only reduces steric hindrance due to the R³ group (Scheme 2), but also forms a secondary amide bond which is the basis of peptidomimetic compounds.⁴⁷ Additionally we wish to compare the yields obtained when employing methanol and 2,2,2-trifluoroethanol as the solvents since significant byproduct formation occurs with the former as described earlier. To this point mainly lipases have been investigated for resolution of Ugi adducts, thus attention in this work will be on the application of EKR to U-4CR enantiomers using protease and penicillin G acylase enzymes.

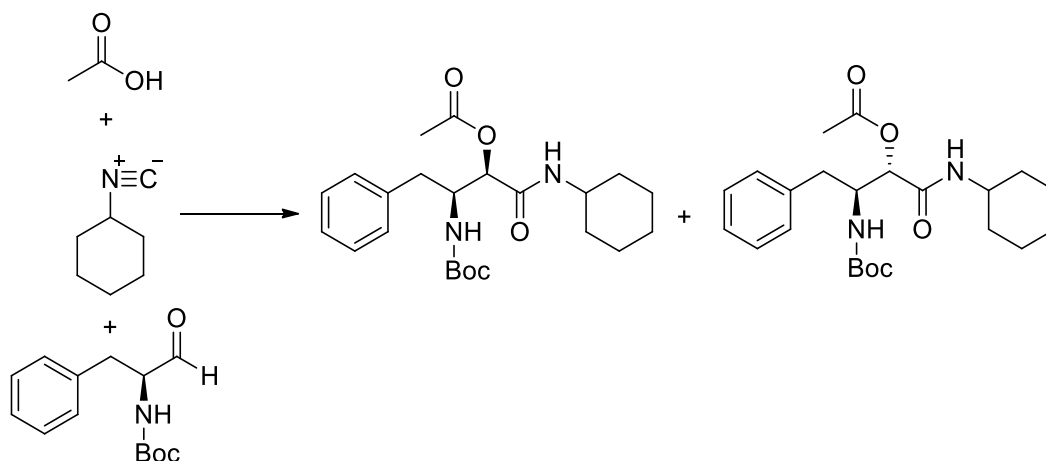
Secondly, EKR of the Passerini reaction starting from achiral starting materials will be investigated using lipases (Scheme 18).



Scheme 18: Proposed enzymatic separation of Passerini enantiomers.

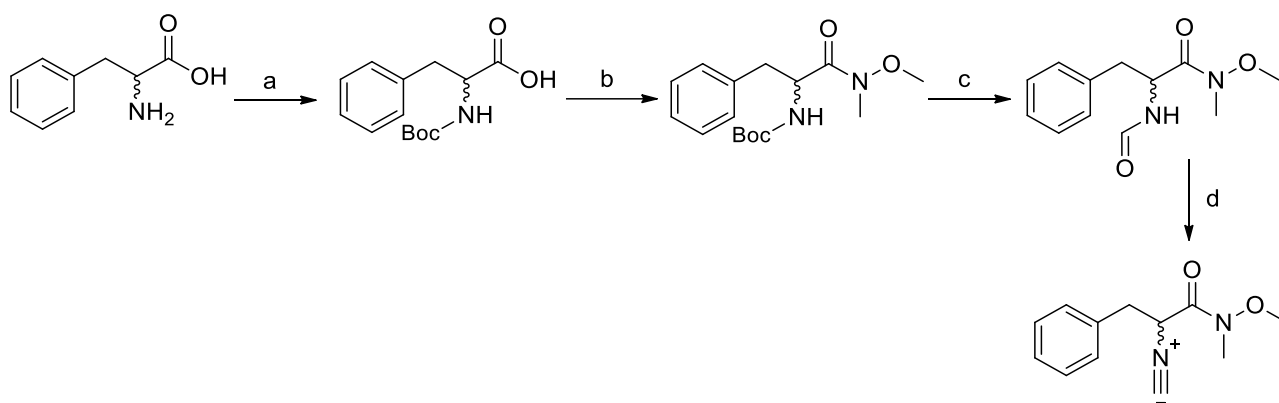
Finally, the diastereoselectivity of the Passerini reaction will be investigated, starting from chiral aldehydes, acids and isocyanides. This is a continuation of work started in our laboratories by Zimuwandeyi.⁸⁰ Zimuwandeyi made a library of compounds using a chiral aldehyde with achiral cyclohexyl-, xyl- and adamantyl isocyanides and achiral acids. The diastereomeric ratios of the Passerini products were approximately 2:1 in each case (Scheme 19). On the other hand, using a chiral aldehyde with an isocyanide that also contains a stereogenic centre (phenylalanine isocyanide)

reduced the diastereomeric ratio to roughly 1.5:1.⁸⁰ This preliminary data clearly indicated that the stereoselectivity of the Passerini reaction could be affected by the introduction of chiral starting material. Thus, our aim will be to fully investigate the reaction diastereoselectivity using chiral aldehydes, isocyanides and acids.



Scheme 19: Diastereomeric Passerini reaction performed by Zimuwandeyi.

The reagents will be synthesised from the commercially available amino acids, D- or L-phenylalanine. The Boc-protected version of phenylalanine will serve as the carboxylic acid component of the reaction. *N*-Boc-D- or L-phenylalaninal can be obtained from reduction of the Weinreb amide of *N*-Boc-phenylalanine (Scheme 20). An extension of this synthetic route can be used to synthesise D- or L-phenylalanine isocyanide (Scheme 20). Adamantyl isocyanide, which does not possess a stereogenic centre will also be prepared via formylation of adamantyl amine, followed by dehydration to the desired product.



Scheme 20: Reagents: a) $(\text{Boc})_2\text{O}$, 1,4-dioxane, KOH b) CDI, CH_2Cl_2 , $\text{NH}(\text{OMe})\text{Me} \cdot \text{HCl}$ c) HCOOH , Ac_2O , TFA d) PPh_3 , CCl_4 , DIPEA, CH_2Cl_2 .

1.5 Aims of the project

Peptidomimetic compounds offer inventive solutions to the drawbacks posed by the use of natural peptides as drugs. MCRs present an efficient route to synthesising such compounds, which in combination with EKR provides the most eco-friendly approach to obtaining compounds with prospective biological activity. Hence the aims of this project are:

- Synthesis of peptide mimics using the U-4CR with employment of ammonia to achieve enantiomers and resolution of the racemic mixtures by proteases and penicillin G acylase.
- Synthesis of Passerini enantiomers alongside corresponding hydrolysis standards and resolution of the products using lipases.
- Synthesis of chiral reagents for use in the Passerini reaction to generate diastereomers and analysis of reaction stereoselectivity followed by separation of the diastereomeric mixtures by preparative chromatography.

Chapter 2: Results and Discussion

As per the aims discussed in Chapter 1, this project was divided into three main sections. The first part (2.1) details the attempt at using EKR for the resolution of enantiomers obtained from the Ugi reaction. Section 2.2 describes the synthesis of Passerini enantiomers and the biocatalytic resolution thereof. The enantioselectivity of the enzymatic reactions is also examined. The final section, 2.3, will centre on the diastereoselectivity of the Passerini reaction when a range of chiral starting materials are employed in this MCR.

2.1 Attempted Enzymatic Kinetic Resolution of U-4CR Enantiomers

2.1.1 Preparation of Ugi Enantiomers

The objective of this section of the project was to prepare peptidomimetic compounds using the Ugi-four component reaction. The racemic mixture resulting from this was intended to be separated by dint of EKR to produce enantiopure adducts that could potentially display biological activity.

There have not been remarkable successes in the resolution of diastereomers from MCRs employing biocatalysts.⁸⁰ Hence we opted first to synthesise Ugi enantiomers which perhaps could deliver more promising results.

We envisaged synthesising a library of U-4CR analogues using readily available starting materials. Benzaldehyde, cyclohexyl isocyanide and ammonia (as the amine element) were chosen as the fixed reagents, with five different carboxylic acids as shown in Figure 7. The reaction was initially performed in methanol as the solvent, as is commonly done with the Ugi reaction. However this has a negative effect on the yields which will be discussed later.

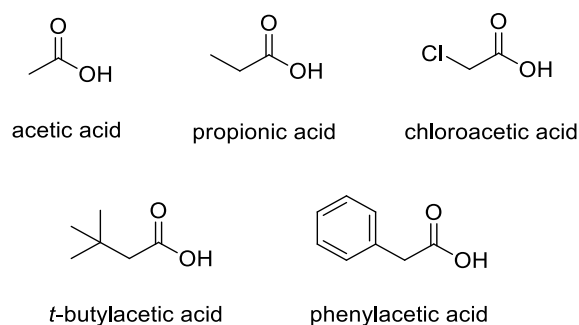
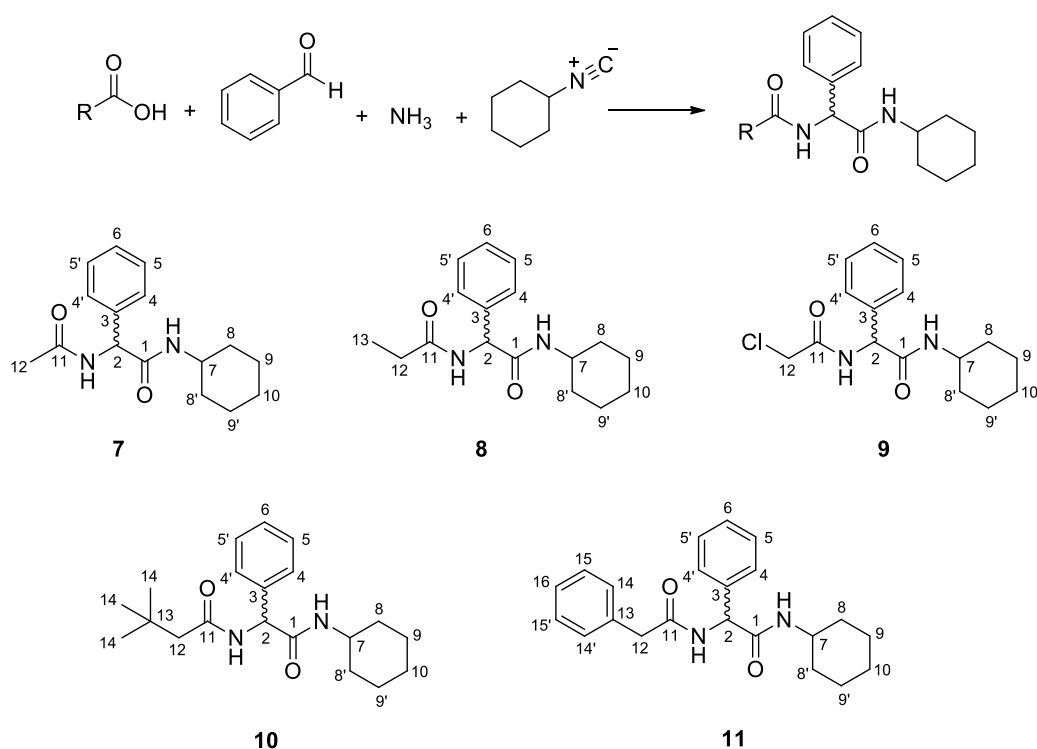


Figure 7: Commercially available carboxylic acids used in the U-4CR.

The U-4CR compounds, **7** - **11** (Scheme 21) were prepared by dissolving the aldehyde in a solution of ammonia in methanol. After 15 minutes of stirring, the acid was added followed by the isocyanide. This mixture was allowed to react overnight at room temperature. From thin layer chromatography (TLC) analysis, the reaction mixture did not show any distinct spots which could conceivably correspond to the desired compound, but rather a long streak that ran across the entire length of the TLC plate. This indicated that there were several products that resulted from the reaction, which were all similar in polarity and hence difficult to separate, even with a variety of solvents used as the mobile phase. Purification of the compounds by column chromatography proved to be challenging as approximately five different compounds were eluted, excluding starting material (cyclohexyl isocyanide which was viewed by use of an iodine stain, and benzaldehyde). The mobile phase which was a mixture of methanol and dichloromethane, had to be adjusted gradually and cautiously to allow compounds to elute from the column separately, especially since the R_f values of the anticipated products were not initially known.

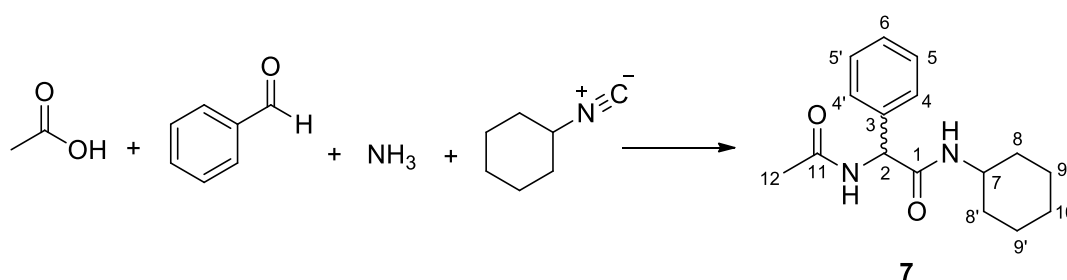


Scheme 21: General synthesis of the U-4CR compounds **7** - **11**.

The NMR spectra were obtained for all five eluted compounds to ascertain if the expected Ugi compound was successfully synthesised. Fortunately the products had been formed as a racemic

mixture, but predictably in low yields ranging from 3 – 18%. There have been numerous reports on the low yields experienced when using ammonia and methanol in the U-4CR, owing to extensive byproduct formation due to the involvement of the solvent in the reaction. In our hands, four different side products were obtained. Although **2** (Figure 4) was found to be the most common byproduct from these reactions as stated by Pick and co-workers,⁴⁷ the NMR data from the unwanted compounds we obtained was indecipherable and could not be characterised as any noteworthy products.

The ¹H NMR spectra of the desired U-4CR compounds did however confirm their presence, although only small amounts were produced. The spectroscopic data for all the U-4CR compounds, **7- 11** were essentially similar except for the R groups (Scheme 21) which differ due to the carboxylic acid involved. Starting with **7** which was prepared using acetic acid (Scheme 22), the ¹H NMR spectrum contained 8 signals. Proton peaks were observed for the phenyl ring stemming from benzaldehyde at 7.47 – 7.15 ppm which integrated for 5 protons as a multiplet. The cyclohexyl ring exhibited 2 peaks in the range of 1.97 – 0.88 ppm for the five CH₂ groups both in the axial and equatorial position and a multiplet peak for H-7 at 3.84 – 3.50 ppm. The R-group, CH₃ from acetic acid exhibited a singlet at 2.01 ppm which is in the expected region for protons of this moiety. The most valuable feature of the ¹H NMR spectrum is the signal at 5.53 – 5.37 ppm which corresponds to H-2 of compound **7** at the new stereogenic centre generated from the reaction. The signal integrates for 1 proton split into a multiplet. Another important indication which proves the formation of the U-4CR **7** are the two NH signals which result from the two amide bonds characteristic of the α-acylaminocarboxamide product. The exact peaks corresponding to a particular NH could not be ascertained, but both signals appear as doublets at 6.97 and 5.86 ppm with *J* = 6.8 and 8.0 Hz, respectively. The multiplicity is most likely owing to coupling with H-2 and H-7 which are the nearest protons to the NH groups.



Scheme 22: Synthesis of **7** with acetic acid. Reagents and conditions: MeOH, room temperature, 24 h.

Significant observations for **7** from the ^{13}C NMR spectrum include two amide carbon atoms at 169.62- and 169.12 ppm, the newly formed stereogenic centre C-2 at 56.90 ppm and C-12 at 23.20 ppm for the R group.

Compounds **8** – **11** display analogous peaks for the functional groups that are identical in both the ^1H and ^{13}C NMR spectra. Unfortunately the cyclohexyl CH_2 proton signal at 1.89 – 0.81 ppm of **11** integrates for more than the 10 expected protons, potentially due to impurities. The R groups from each of the acids used appear at the expected chemical shifts and verify that the Ugi compounds were successfully yielded. In addition the ^{13}C NMR spectrum of **8** contains doubled signals for C-12 at 29.72- and 29.52 ppm, indicating the presence of rotamers for this compound.

Another indication of the presence of amide bonds was obtained by infrared spectroscopy. The NH stretch occurs at 3280 cm^{-1} and a strong C=O stretch appears at 1633 cm^{-1} for the U-4CR product **7**. The other Ugi compounds display comparable results and are listed in Table 1.

Table 1: Selected IR signals for Ugi compounds **8** – **11**.

Compound	8	9	10	11
NH stretch (cm^{-1})	3283	3291	3295	3294
C=O amide stretch (cm^{-1})	1634	1633	1628	1632

High resolution mass spectrometry was also carried out for all the compounds and support the other data to prove effective preparation of **7** – **11**.

In addition, the U-4CR was also performed using butyric, formic and benzoic acid, and no products were isolated, although a number of uncharacterisable byproducts were collected.

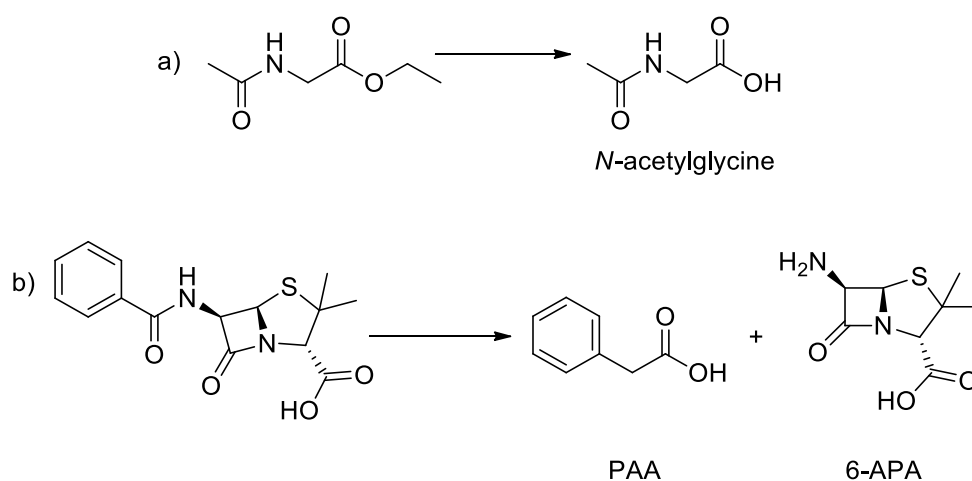
Although five Ugi compounds were successfully obtained, the poor yields and problematic purification were still a concern which needed to be addressed. The apparent issue was the use of ammonia combined with methanol. From earlier reports, Thompson and Chen improved the yields of Ugi compounds by employing 2,2,2-trifluoroethanol as the solvent as it is less nucleophilic and less likely to participate in the reaction, causing side reactions.⁴⁰ We decided to repeat the preparation of **7** and **10**, which were achieved with acetic and *t*-butylacetic acid, respectively, using 2,2,2-trifluoroethanol. The results were excellent as the yield for **7** improved from 12% to 40%, and from 18% to 70% for compound **10**. This approximated to an increase of more than threefold, which is even greater than

that achieved by Thompson and Chen.⁴⁰ Nevertheless, compound **11** was obtained in an unsatisfactory yield of 14% despite the fact that it was synthesised using 2,2,2-trifluoroethanol from the outset.

2.1.2 Attempted EKR of **7** and **11**

After attaining the U-4CR enantiomers, we sought to subject the compounds to biocatalytic resolution. Although there have been past reports of EKR of Ugi compounds with lipase enzymes, no known work has been done using proteases. This presented an opportunity for our research into EKR of Ugi enantiomers via protease enzymes.

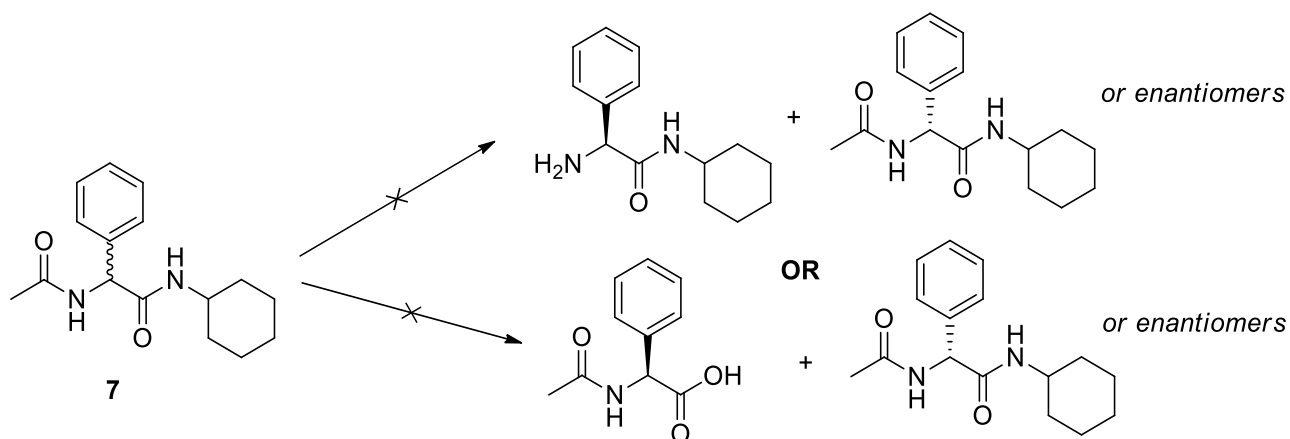
Before subjecting the compounds to enzymatic reactions, the activity of the proteases had to be established. The assay was performed with *N*-acetylglycine ethyl ester dissolved in phosphate buffer of pH 7.5 at 40 °C. A control reaction was set up concurrently to affirm that the compound was not being hydrolysed without the enzyme. Five proteases (Alcalase, *B. clausii* protease, *B. lentus* protease, *Bacillus* species protease and *B. licheniformis* protease) were tested and all proved active by hydrolysing the ester bond of the substrate to produce *N*-acetylglycine (Scheme 23).



Scheme 23: a) Activity assay of proteases using *N*-acetylglycine ethyl ester b) Activity assay of penicillin G acylase with the substrate Penicillin G.

In addition to proteases, we also investigated penicillin G acylase for EKR. This enzyme specialises in cleaving phenylacetylated compounds at the amide bond, making it more substrate-specific which is also the reason pharmaceutical industries are highly attracted to it for antibiotic production.⁸¹ On

account of this, the Ugi product **11** which incorporated phenylacetic acid (PAA) was prepared to allow hydrolysis by penicillin G acylase at the target bond. The activity of this enzyme was also determined by investigating the hydrolysis of the substrate Penicillin G to generate 6-aminopenicillanic acid (6-APA) and PAA (Scheme 23). The assay was conducted under the same conditions as the proteases and also tested active.



Scheme 24: Reagents and conditions: Protease enzyme, phosphate buffer pH 7.5, DMSO/ MBE, 40 °C.

Henceforward we were able to perform the biocatalytic reactions on the Ugi compounds. We resolved to attempt EKR with the proteases on compound **7** first (Scheme 24) as it possessed the least bulky R-group from acetic acid thus making it easier for enzymes to access, as compared to **8 – 11** which were synthesised using larger acids. We predicted that the enzymes would cleave the amide bond at the acetyl group of **7**, but it was also possible for it to hydrolyse the cyclohexyl-containing amide bond as presented in Scheme 24. Compound **7** was insoluble in the aqueous buffer and needed to be dissolved in an aqueous-miscible organic solvent in order for the substrate and enzyme to be in the same phase, to increase the probability of a reaction taking place. This posed a problem since the substrate showed extremely poor solubility in roughly 40 different solvents (listed below). For a few of the solvents in which the compound proved marginally soluble when heated, the compound precipitated instantly out of solution when the buffer was added. DMSO was the only water-miscible solvent in which **7** dissolved, but it also precipitated out of solution in the buffer when large amounts of the compound were used. Hence the reaction was done with only 10 mg/ml in DMSO, and this was further diluted in a 0.2:1 volume ratio of solvent-substrate solution to aqueous buffer. A set of reactions was set up using each of the active proteases in generous quantities. TLC was the first choice of analysis method to monitor any transformation by the enzyme. A new spot was seen after 48 hours with enzyme FE202

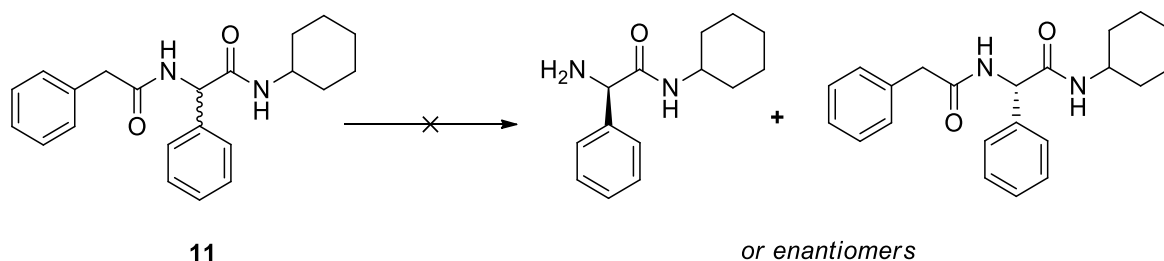
(in a vanillin staining solution), but after obtaining the NMR and mass spectrometry results, none of the expected hydrolysed product was present and only starting material was isolated. Regardless of this, the reactions were repeated and left to stir for 30 days with no indication of hydrolysis and the experiments were then terminated.

Organic solvents tested for solubility of compounds 7 and 11:

- | | |
|----------------------------|-----------------------------------|
| 1. 1,4-Dioxane | 21. 2-Methyl-but-3-ene-2-ol (MBE) |
| 2. Acetone | 22. Glycerine |
| 3. Ethyl acetate | 23. Ethanol amine |
| 4. Diethyl ether | 24. Iso-butyl alcohol |
| 5. Petroleum ether | 25. Amyl alcohol |
| 6. Hexane | 26. 1,2-Dimethoxy ethane |
| 7. Methanol | 27. Benzyl alcohol |
| 8. DMSO | 28. Methyl ethyl ketone |
| 9. Water | 29. Phenyl acetate |
| 10. Ethanol | 30. Diisopropyl ether |
| 11. <i>n</i> - Butyl ether | 31. Chloroform |
| 12. Propan-2-ol | 32. Dichloromethane |
| 13. DMF | 33. THF |
| 14. Xylene | 34. Di- <i>n</i> -butyl phthalate |
| 15. <i>n</i> - Heptane | 35. Diphenyl ether |
| 16. Toluene | 36. Octanol |
| 17. 2-Butanol | 37. 3-Ethyl hexanol |
| 18. Cyclohexane | 38. Ethylene glycol diethyl ether |
| 19. Acetic anhydride | 39. Acetonitrile |
| 20. 2-Methyl-propan-2-ol | |

One other solvent that **7** was able to dissolve in was 2-methyl-but-3-ene-2-ol. This also had limitations as only small amounts of substrate could be added to the solvent for it to remain in solution, as well as MBE being immiscible with the phosphate buffer, hence the EKR reactions were performed as a two-phase reaction. After 14 days, this system also showed no positive outcome and all five of the proteases failed once more to hydrolyse any of the amide bonds of the Ugi compound **7**. Because of the poor solubility of the product, we were unable to track the enzymatic reactions using HPLC to gain

a more accurate result. The two solvents, DMSO and MBE, which were used were not suitable to inject into HPLC chiral columns so we resorted to TLC, NMR and mass spectrometry for analysis.



Scheme 25: Reagents and conditions: Penicillin G acylase, phosphate buffer pH 7.5, EtOH, 40 °C.

Following the disappointing results with **7**, our next endeavour was to resolve the U-4CR **11** with penicillin G acylase, in the hope that it would more likely hydrolyse the amide bond at the phenylacetic acid group, as it does for the natural substrate (Scheme 25). We began by assessing the solubility of the compound, and noticed that from the solvents listed above only ethanol dissolved the substrate, and only to a certain degree. Akin to substrate **7**, the experiment was conducted using a mere 10 mg/ml solution of **11** in ethanol in the aqueous buffer at 40 °C. The reaction was monitored using the same techniques applied to the previous substrate, and likewise after 30 days, no reaction of any kind was observed. We postulated that even if a larger quantity of the compound was not completely soluble in the solvent at the beginning of the reaction, an enzymatic reaction could still occur as the mixture turns into a solution after some time of vigorous stirring. Consequently we ran a further experiment, this time using a larger quantity of compound. Within a number of days of agitation, the compound dissolved in the ethanol-buffer system, but still no enzymatic transformation had taken place. The reaction was again terminated after 30 days.

With a great deal of time spent optimising the enzyme reactions, attempts at EKR on the Ugi enantiomers, particularly **7** and **11**, yielded no favourable outcome. Failure of biocatalytic resolution could be due to several reasons, the most likely thought to be structural incompatibility between the U-4CR adducts and the enzymes concerned. Compound **7** could possibly be too bulky, causing steric hindrance, as seen in work completed by Zimuwandeyi on the Passerini reaction.⁸⁰ Since penicillin G acylase is proficient in cleaving PAA chains, it is most likely other parts of the structure of **11** that are not suitable. It could be that the functional groups on the compound are not able to form non-covalent

bonds with the amino acid residues of the active site, leading to no reaction. Another cause of the lack of enzyme activity could also be the poor solubility of both the compounds, making it a greater challenge for the biocatalysts to come into close contact with the substrate. In brief, EKR of the enantiomers generated from the Ugi MCR was unsuccessful, thus investigation of resolution of Ugi diastereomers seemed futile at this point. Therefore we strove to separate peptidomimetic compounds from a similar MCR, the Passerini reaction, by means of enzymes which will be discussed in the following section.

2.2 Separation of Passerini Enantiomers by EKR

2.2.1 Synthesis of Passerini Enantiomers

The Passerini reaction was performed with three different commercially available aldehydes as presented in Figure 8, using acetic acid and cyclohexyl isocyanide as the other reaction components. Cyclohexyl isocyanide was chosen as it is economical and more accessible in comparison to other isonitriles. The reaction was conducted using equimolar amounts of each of the reagents dissolved in CH_2Cl_2 with stirring for 3 days at room temperature. TLC indicated formation of a new product, as well as starting material. Purification by column chromatography was much less problematic than with the U-4CR products and led to the desired Passerini products **12**, **13** and **14** (Scheme 26) as a racemate in average yields of 61-, 44- and 57%, respectively.

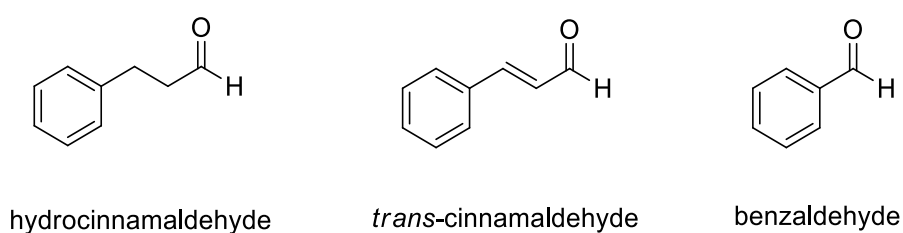
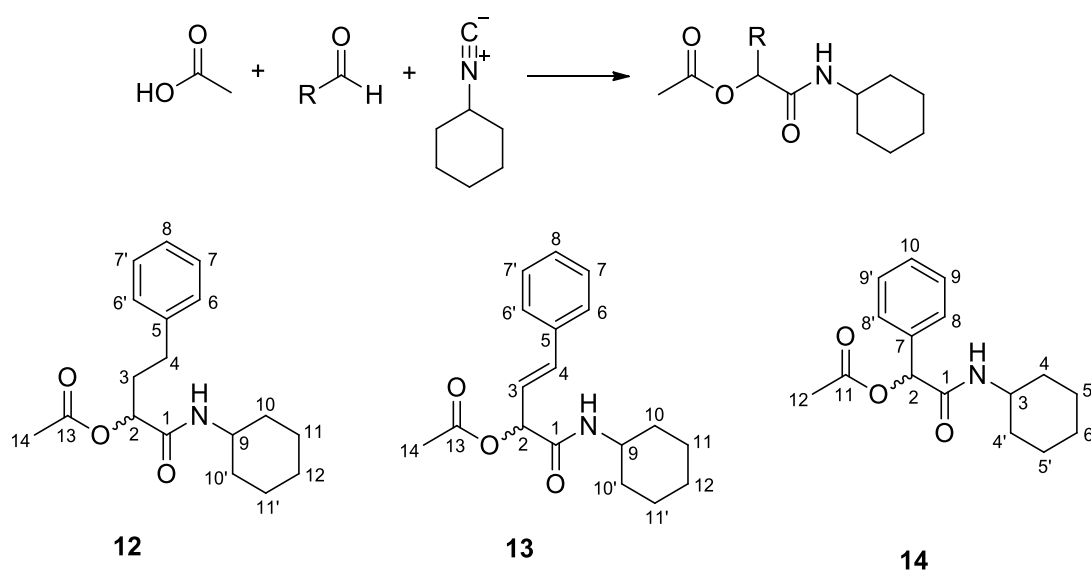


Figure 8: Commercially available aldehydes used in the Passerini MCR.



Scheme 26: General synthesis of the Passerini compounds **12** - **14**.

The formation of **14**, which was synthesised using benzaldehyde, was verified spectroscopically. The ^1H NMR spectrum showed a multiplet at 7.56 – 7.20 ppm integrating for 5 protons which corresponds to the aromatic protons of the aldehyde and a singlet was observed at 2.10 ppm for the acetyl CH_3 group. Another clear indication of the success of the reaction is emphasised by the signal at 6.02 ppm from the stereogenic centre for H-2, in addition to the NH peak which appears at 6.76 – 6.55 ppm attributed to the new amide bond created. Twelve distinct signals were observed in the ^{13}C NMR spectrum as predicted. Downfield signals at 169.16 and 167.26 ppm were ascribed to the carbonyl groups from both the ester and amide bonds. The peak for the stereogenic carbon, C-2 was seen at 75.54 ppm. The most shielded carbon atom, C-12, was characterised by the signal at 21.03 ppm. The characterisation data obtained for **14** corresponded to that reported in the literature for the same compound.⁸²

Compounds **12** and **13** were prepared using hydrocinnamaldehyde and *trans*-cinnamaldehyde, respectively, hence their structures are similar aside from the alkene bond of **13**. For this reason the NMR data are also similar as shown in Table 2. The major disparities arise at the signals for H-3 and H-4 as well as the proton at the stereogenic centre. H-3 and H-4 consist of two CH_2 groups for **12**, which are signalled in the ^1H NMR spectrum as an atypical singlet at 2.10 ppm (expected to appear as a multiplet) and a triplet with $J = 7.9$ Hz at 2.67 ppm, respectively. However, compound **13** comprises of two CH groups at the double bond, with H-3 displaying a peak at 6.27 ppm and H-4 at 6.73 ppm, both

of which have the multiplicity of a doublet of doublets. The stereogenic centre proton, H-2, is located more downfield at 5.71 ppm for **13** owing to deshielding by the alkene group, as contrasted with H-2 from **12** which occurs at an upfield shift of 5.13 ppm. The multiplicity of H-2 for **13** is likewise a doublet of doublets, indicating probable coupling of H-2 with both H-3 and H-4.

The ^{13}C NMR spectra support these differences showing a peak at 122.73 ppm for C-3 and another at 135.72 ppm for C-4, representative of the C=C bond of product **13**. More upfield signals for **12** are observed, with C-3 at 33.32 ppm and C-4 at 31.12 as anticipated. The position of the C-2 signals for **12** and **13** were very similar as opposed to the significant difference in the chemical shifts of H-2 observed in the ^1H NMR spectra of these compounds. Compound **13** exhibited a peak at 74.5 ppm and **12** a peak slightly upfield at 73.75 ppm for C-2.

Table 2: Selected NMR shifts for identical groups of Passerini compounds **12** and **13**.

	Compound 12		Compound 13	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
Aromatic region	δ 7.36 – 7.08 (5H, m)	δ 140.85 - 126.11	δ 7.46 – 7.19 (5H, m)	δ 134.72 - 126.83
NH	δ 5.86 (1H, d, J = 8.0 Hz)	---	δ 5.89 (1H, d, J = 7.9 Hz)	---
H-3 / C-3	δ 2.10 (2H, s)	δ 33.32	δ 6.27 (1H, dd, J = 15.9, 6.8 Hz)	δ 122.73
H-4 / C-4	δ 2.67 (2H, t, J = 7.9 Hz)	δ 31.12	δ 6.73 (1H, dd, J = 16.0, 0.9 Hz)	δ 135.72
H-9 / C-9	δ 3.87 – 3.69 (1H, m)	δ 48.02	δ 3.89 – 3.69 (1H, m)	δ 48.26
H-14 / C-14	δ 2.17 (3H, s)	δ 20.92	δ 2.20 (3H, s)	δ 21.07
Cyclohexyl CH₂	δ 1.97 – 1.05 (10H, 3xm)	δ 30.91 - 24.78	δ 1.98 – 1.05 (10H, 4xm)	δ 33.02 - 24.78

Analysis of the infrared spectra furthermore shows strong amide and ester stretches indicative of the new functional groups generated by the Passerini reaction to achieve compounds **12** – **14**. The precise positions of the signals are contained in Table 3. Compound **12** is novel, however the literature data obtained for compound **13** matched the characterisation data that we obtained for **13**.⁸³

Table 3: Key IR signals that confirm the formation of Passerini products **12** – **14**.

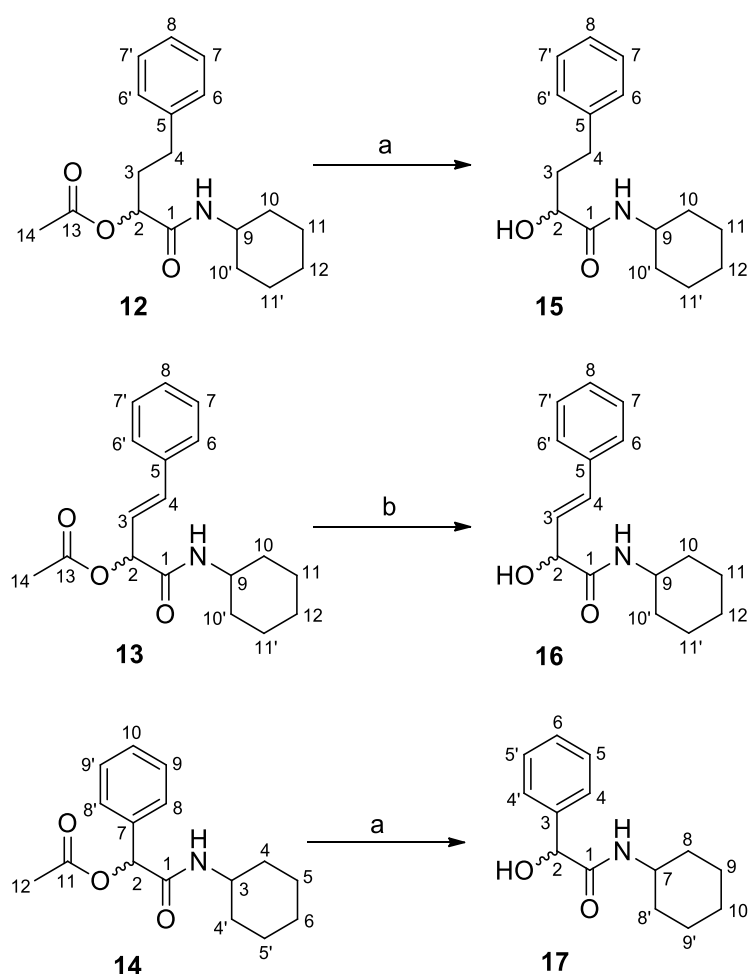
Compound	12	13	14
NH stretch (cm ⁻¹)	3284	3289	3318
C=O ester stretch (cm ⁻¹)	1740	1733	1738
C=O amide stretch (cm ⁻¹)	1650	1661	1660

The melting points for compounds **13** and **14** were found to be equal at 123 – 125 °C, whereas **12** displayed a lower value of 100 – 103 °C conceivably due to more aliphatic groups contained within the substrate. The melting point for **13** found in the literature was 132 – 133 °C which is notably similar to what we obtained.⁸³

2.2.2 Preparation of the Hydrolysis Standards for EKR

With the Passerini enantiomers in hand, we could proceed with the synthesis of the hydrolysed compounds. Since we planned on resolving the Passerini compounds via lipase hydrolysis, it was necessary for us to prepare standards for the reaction as we set to pursue the EKR studies by way of HPLC, which will be expanded on later.

Accordingly we chemically hydrolysed the ester functionality of the Passerini compounds **12** – **14** as illustrated in Scheme 27.



Scheme 27: Reagents and conditions: a) KOH, MeOH, 24 h b) 1.5 M H₂SO₄, EtOH, 90 min.

Compounds **12** and **14** were hydrolysed under basic conditions. The starting material was treated with 10 equivalents of KOH in a methanolic solution and the reaction continued for 24 hours. Following the work up and extraction with ethyl acetate, both **15** and **17** were furnished as cream-white solids, as racemic mixtures in yields of 51 and 75%, respectively. No further purification was required. The *R_f* values of the products were also lower than for the corresponding starting materials attributed to the more polar OH group formed at the stereogenic centre.

The basic hydrolysis method was also initially performed on **13**, but the desired compound was not produced. We postulated that another reaction, probably allylic substitution, was taking place at the double bond rather than the desired reaction at the ester moiety. Thus we adopted a hydrolysis method using acidic conditions. Compound **13** was reacted with 1.5 M H₂SO₄ in ethanol. After 90

minutes of slight heating and extraction using ethyl acetate, column chromatography was undertaken and **16** was successfully obtained in a good yield of 74% as a yellow solid.

The ^1H NMR spectra displayed significant changes for the hydrolysed products in comparison to the Passerini compounds. The proton peaks at ~ 2.1 ppm associated with $\text{CH}_3\text{C}=\text{O}$ at the ester functionality of **12** – **14** no longer appeared in the spectra of **15** – **17**, validating effective hydrolysis of the ester bond. The signals for CH at the stereogenic centre shifted significantly upfield, with changes also seen for the NH peaks. For instance H-2 of **15** is observed at 4.08 ppm, whereas in the starting material it arises at 5.13 ppm. The NH peak shifted downfield from 5.86 ppm to 6.72 ppm in the product. This pattern also follows through in the ^1H NMR spectra of compounds **16** and **17**.

The ^{13}C NMR data is also convincing as there were two carbonyl peaks at $\sim 168/169$ ppm for the Passerini products, but the hydrolysed compounds only exhibited one C=O peak due to the amide bond, proving that hydrolysis of the ester group occurred. Another clear confirmation is the absence of the CH_3 signal which normally arose at ~ 21 ppm, from all three hydrolysed adducts. Moreover, a minor change in shift value for the stereogenic carbon atom to a more upfield position was noticed, for example **15** exhibited a C-2 peak at 71.38 ppm in comparison to 73.75 ppm for **12**.

The major peaks observed in the infrared spectra for **12** – **14** are shown in Table 4. Broad peaks correlating to the OH stretches are present (Table 4) which are not seen for the starting material. The ester C=O stretch at $\sim 1740\text{ cm}^{-1}$ for **12** - **14** as shown in Table 3 is also non-existent in the products, once more substantiating successful hydrolysis of the Passerini compounds.

Table 4: Major IR peaks of the Hydrolysis Standards.

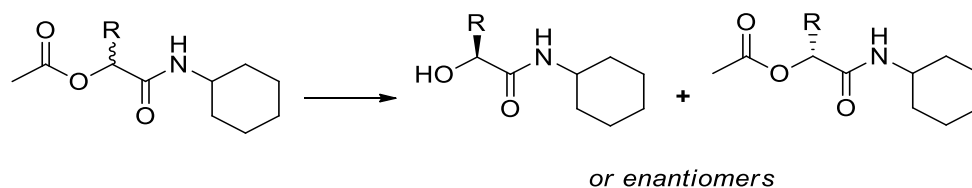
Compound	15	16	17
NH stretch (cm^{-1})	3284	3395	3335
OH stretch (cm^{-1})	3278	3272	3251
C=O amide stretch (cm^{-1})	1635	1636	1637

The mass spectra of **15** – **17** were furthermore consistent with the expected masses of the standards. All the characterisation data obtained for compounds **15** – **17** corresponded to that obtained in the literature, proving that the synthesis of these compounds were successful.⁸³⁻⁸⁵

2.2.3 Enzymatic hydrolysis of the Passerini enantiomers **12** – **14**

Lipases have been shown to be useful biocatalysts in EKR of secondary alcohols and thus lipases were tested in the EKR of the Passerini enantiomers **12**, **13** and **14**.

The lipases chosen to carry out the reactions were lipases 6Y (Cat # ESL-001-01, Lot # 6Y0240) and 6Z (Cat # ESL-001-01, Lot # 6Z0248), which were purchased from Recombinant BioCatalysis, as they were found in our laboratories to respond well to a range of organic molecules tested. These enzymes were identical except that they were prepared in different lots. An activity assay was also conducted with the enzymes before continuing with the Passerini substrates. Benzyl acetate was used as the test compound in acetonitrile and phosphate buffer. After 3 hours, the enzymes had hydrolysed the compound to produce benzyl alcohol and thus were sufficiently active to be tested on our products. A control reaction containing no lipase was performed simultaneously to establish that no reaction took place in the absence of an enzyme. Standard substrate tests were also performed to ensure that the enzymes responded to our compounds.



Scheme 28: Proposed EKR of the Passerini enantiomers **12** – **14** by Lipases 6Y and 6Z.

Having effectively synthesised the substrates and confirmed the lipase activity, we were now capable of investigating resolution of compounds **12** – **14** by use of both Lipases 6Y and 6Z as exemplified in Scheme 28. We planned on screening the enzymes with the substrates first to ascertain if a reaction was taking place. As acetonitrile was the solvent of choice for the activity assay, we used it as a starting point to test for compound solubility. Unlike the U-4CR adducts, the Passerini products dissolved in acetonitrile effortlessly, even in higher concentrations. Hence we performed the reaction with a 1:1 ratio of phosphate buffer of pH 7.0 to acetonitrile, using a 1:2 ratio of substrate to enzyme (20 mg: 40 mg). This was stirred at 40 °C for approximately 4 hours, with inspection by TLC throughout. The starting material and hydrolysed standards were spotted on a TLC plate, with the reaction mixture alongside. Roughly 20 minutes from the onset, we were elated to see that a new spot had already

formed with an R_f value that corresponded to the hydrolysed standards, in addition to starting material. This was observed for all three Passerini compounds **12**, **13** and **14** from both lipases. This suggested that the enzymes were seemingly compatible with our substrates and were hydrolysing the ester bonds successfully. The reactions continued for nearly 4 hours and the biocatalysts transformed more of the substrate into the respective hydrolysed compounds. We then terminated the reactions and extracted the organic molecules using ethyl acetate. After concentration, we subjected the residue to HPLC, initially using the reversed phase C-18 column to confirm the positive outcome of the enzyme reaction that was observed from TLC. From this we could also determine the amount of substrate being converted to the product.

This set of compounds dissolved without difficulty in acetonitrile, hence the samples for HPLC analysis were prepared at a concentration of 1 mg of substrate per ml of acetonitrile. Only a 25 μ l portion of the solution was loaded onto the column for separation. Although compounds **13** – **17** were not novel, no information was available concerning a method to achieve baseline separation of these products by HPLC.

We started with a mobile phase of a 50% mix of acetonitrile/water at a flow rate of 1 ml/minute. This did not result in the desired separation. We then varied the ratio of solvents until baseline separation was observed. It was not extremely challenging to achieve separation of the substrate and product on the C-18 column. The optimum chromatograms were those showing elution times of less than 5 minutes for the substrates and products of all three of the Passerini enzymatic reaction mixtures, making it simple to follow the EKR reactions.

From the chromatograms of each of the reactions (Figures 9 -14), it is evident that the enzymes were indeed hydrolysing the Passerini products. Commencing with the enzyme reactions of **12**, Figures 9 and 10 show the appearance of both the substrate and the hydrolysed product. The hydrolysed compound **15** eluted at approximately 4 minutes with a mobile phase of an 80:20 acetonitrile/water mixture. The Passerini substrate **12** had an elution time of ~ 4.9 minutes. Using peak area percentage as a semi-quantitative measure, 58.4% of product **15** was produced, relative to the 41.6% of unreacted substrate, after 4 hours by Lipase 6Y. With Lipase 6Z (Figure 11), the results were virtually identical with 55.6% of product formed.

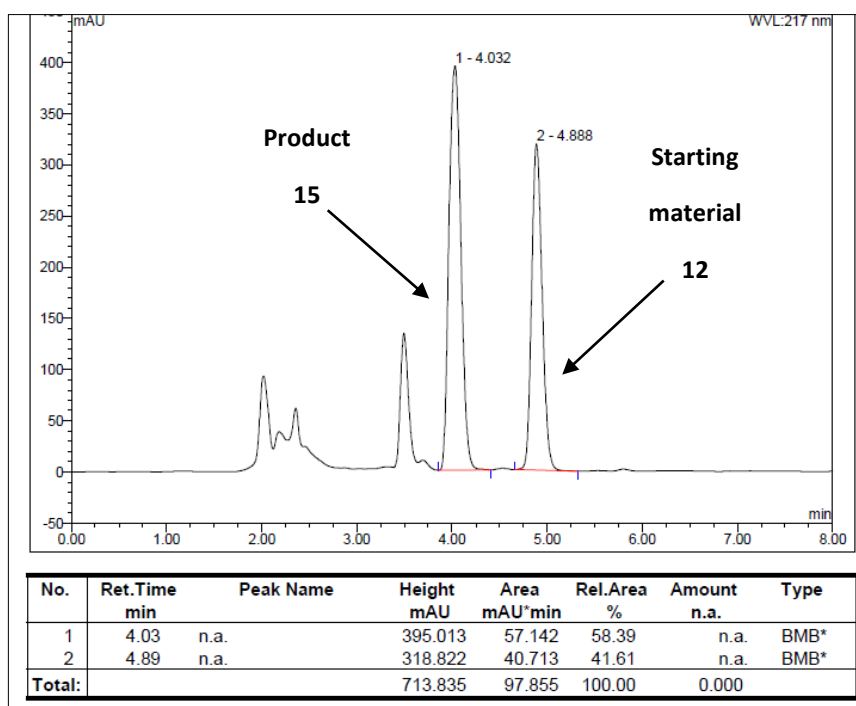


Figure 9: HPLC chromatogram of the enzymatic reaction of **12** with Lipase 6Y after 4 hours.

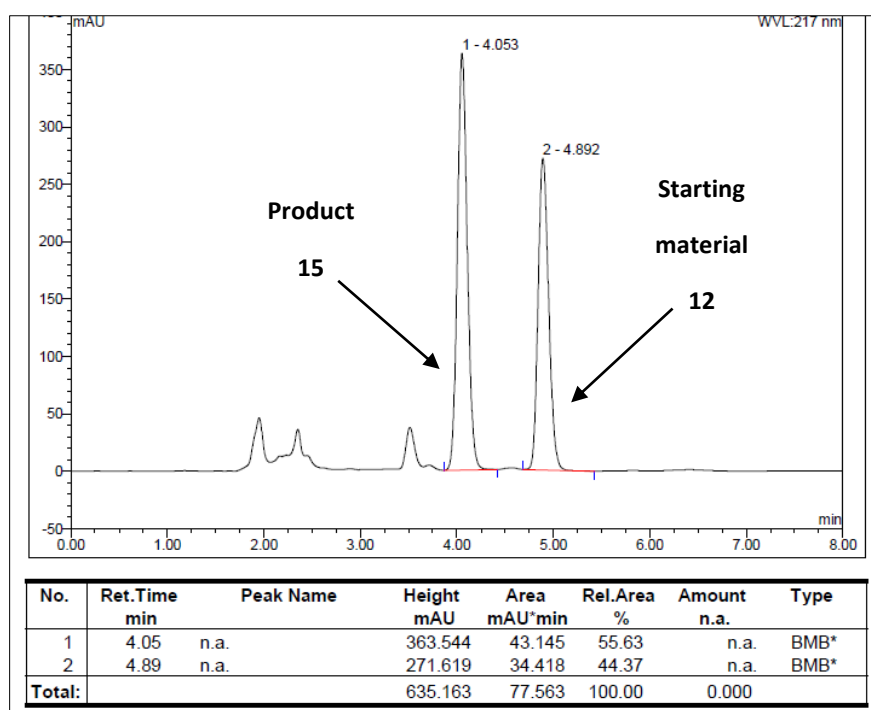


Figure 10: HPLC chromatogram of the enzymatic reaction of **12** with Lipase 6Z after 4 hours.

Maintaining the mobile phase at a ratio of 80:20 acetonitrile/water afforded adequate separation of **13** and **16**. Figures 11 and 12 depict the EKR reactions of the Passerini product **13**. The elution times of the compounds were very similar to those of **12** and **15** which is unsurprising since they are structurally related. Compound **16** was eluted at around 4 minutes and **13** at ~ 4.6 minutes. The rate of conversion of compound **13** was much more gradual in comparison to the previous product. Lipase 6Y produced 18% of **16**, and Lipase 6Z nearly 27% in a span of 4 hours. Although this is considered a positive outcome as the enzymes responded well to the Passerini compound, the rate of hydrolysis of **12** to **15** is more than double that of **13** to **16** for both Lipases 6Y and 6Z. The only observable reason is the less-accessible alkene functionality contained in **13** that potentially causes the biocatalyst to react slower with the compound, and convert less of the substrate to the corresponding product.

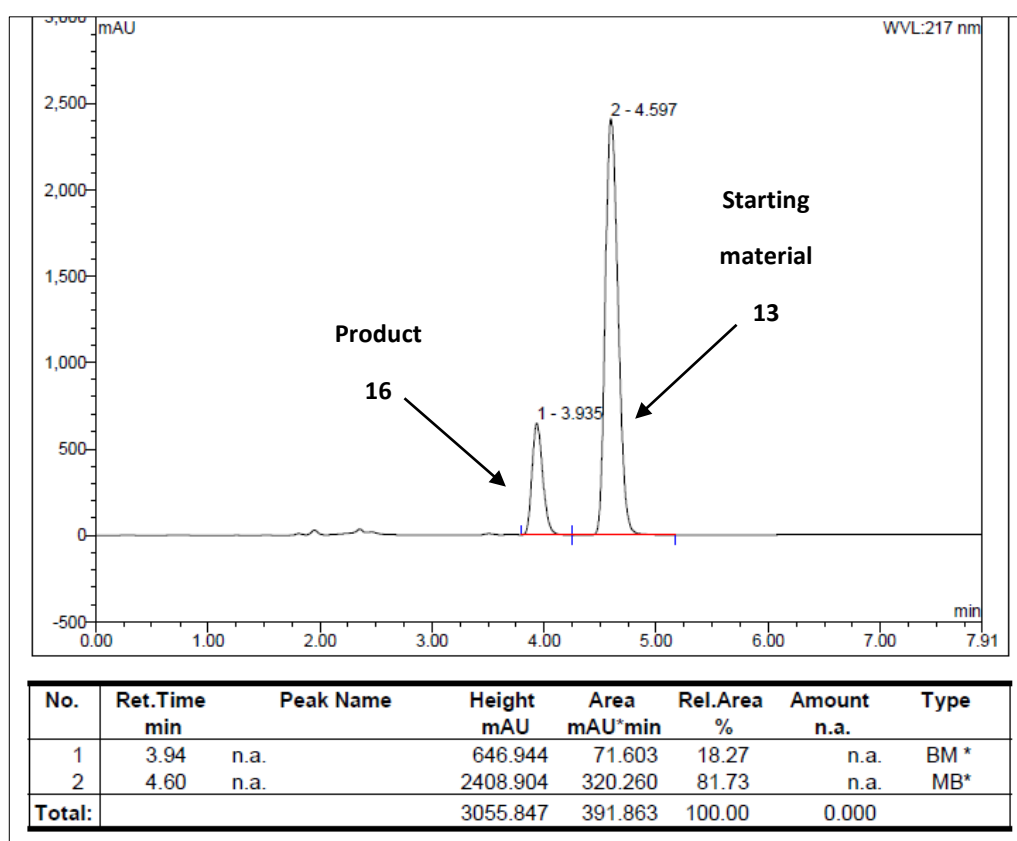


Figure 11: HPLC chromatogram of the enzymatic reaction of **13** with Lipase 6Y after 4 hours.

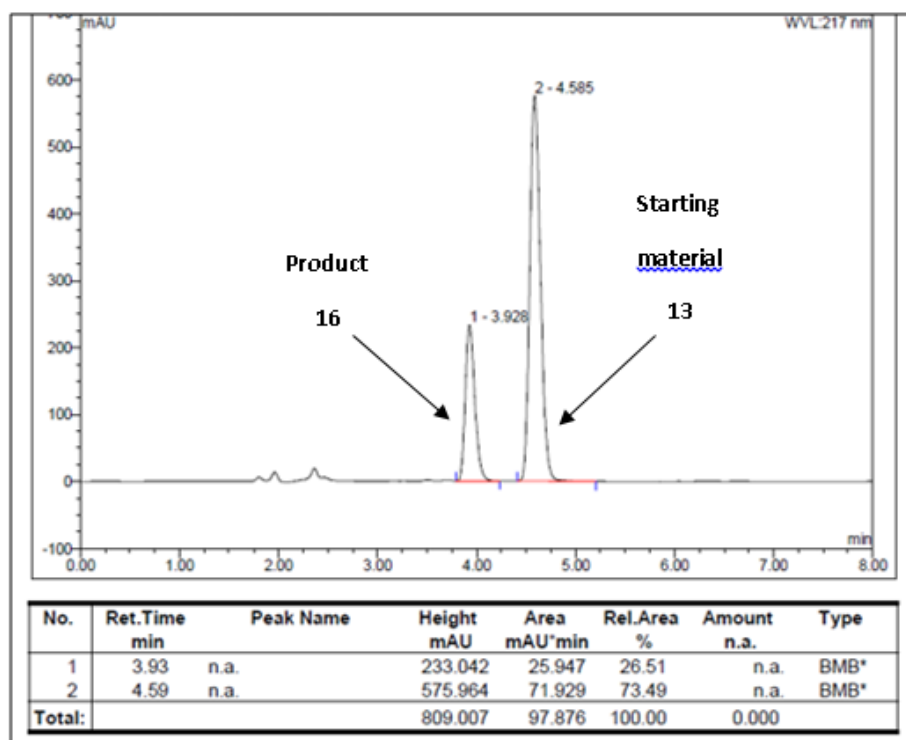


Figure 12: HPLC chromatogram of the enzymatic reaction of **13** with Lipase 6Z after 4 hours.

Separation of **14** and **17** on the C-18 column using the same mobile phase as previously led to even shorter elution times than the preceding adducts. Figures 13 and 14 show that the substrate eluted the column at 4 minutes and the hydrolysed compound even earlier at 3.5 minutes. The enzymatic reactions of compound **14** were more rapid than for the other compounds. At 4 hours, Lipase 6Y had already produced 69% of **17** and Lipase 6Z was not far off with a production of 63%. The rate of these reactions were greater than both the EKR of **12** and **13**. The only distinction between each of the three Passerini adducts are the aldehyde components with which they were synthesised. Compound **14** was prepared with benzaldehyde, which is a simpler, less bulky reagent as compared with hydrocinnamaldehyde and *trans*-cinnamaldehyde used to generate **12** and **13**, respectively. Owing to this, **14** has a structure that is more easily accessible to enzymes, and perhaps fits better in the lipase active site. Hence the rate of hydrolysis is more rapid, affording excellent production of the hydrolysed product contrasted with the other Passerini analogues. Structural compatibility can also rationalise that **13**, synthesised from *trans*-cinnamaldehyde, exhibited the lowest rate of hydrolysis by the lipases due to the complexity of the double bond it possesses. Overall Lipases 6Y and 6Z hydrolysed the set of Passerini compounds at an almost equal rate, aside from compound **13** for which Lipase 6Z had a greater degree of hydrolysis by 10%.

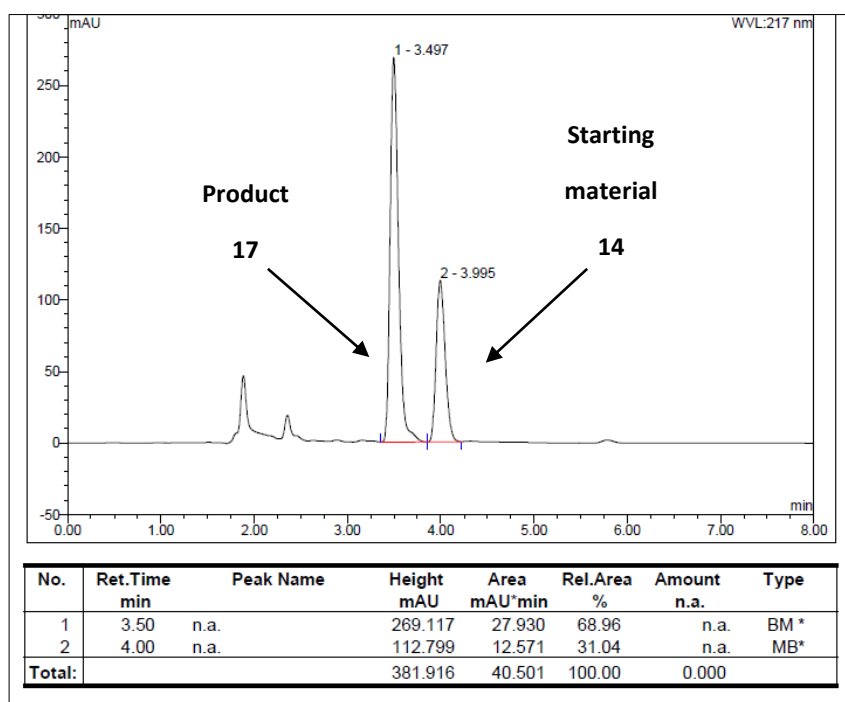


Figure 13: HPLC chromatogram of the enzymatic reaction of **14** with Lipase 6Y after 4 hours.

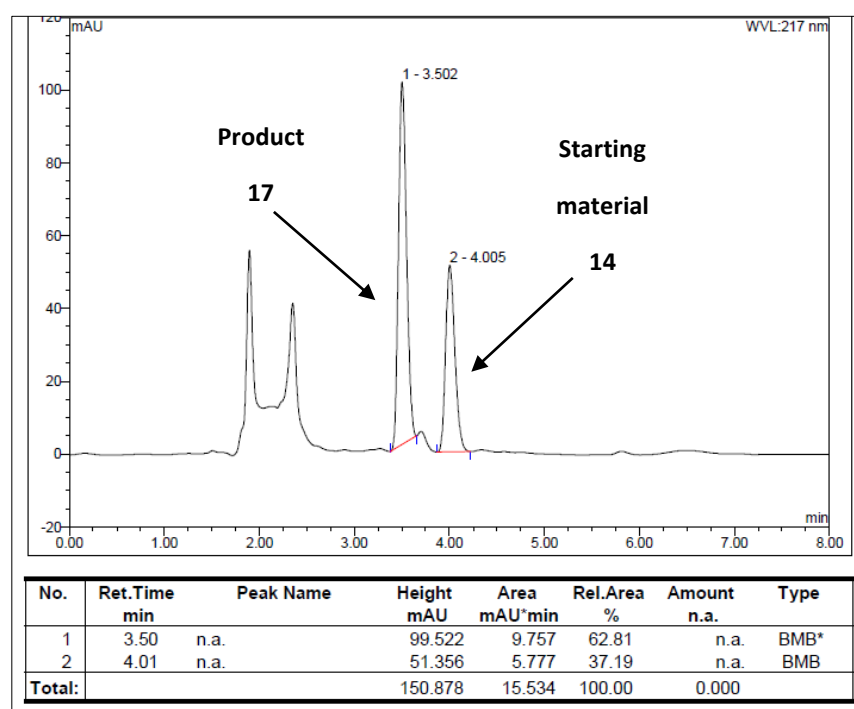


Figure 14: HPLC chromatogram of the enzymatic reaction of **14** with Lipase 6Z after 4 hours.

Analysis by HPLC using the C-18 column provided adequate data to validate the occurrence of hydrolysis by lipases 6Y and 6Z. The conversions of each of the Passerini products were satisfactory. The outcomes of the enzymatic hydrolysis of substrates **12** – **14** were pleasing, and very different from the attempts at EKR on the Ugi compounds. Despite the confirmation of the enzyme reactions, we had yet to discover if the biocatalysts were reacting selectively with one of the two enantiomers, or if they were hydrolysing both enantiomers to the same extent. Hence we explored the enantioselectivity of the reactions by employment of chiral HPLC.

2.2.4 EKR of the Passerini enantiomers

Using the C-18 column we were only able to ascertain the compounds that were present in the enzymatic reaction mixture – specifically the Passerini substrate and the hydrolysed product being formed. However this route is not capable of separating enantiomers, and consequently indicating selectivity of the biocatalyst. Hence it was mandatory for us to subject the reactions to chiral HPLC. This would allow separation of both the substrate racemic mixture and enantiomers of the hydrolysed products, quantitatively. In this way, we could uncover the enantioselectivity of the lipases with regard to the Passerini compounds **12** - **14**.

We repeated the EKR reactions for all three Passerini compounds under the same conditions as mentioned earlier with both Lipases 6Y and 6Z. However, for the purpose of enantioselectivity studies, we removed a portion of the reaction mixture at 20 minute intervals for the duration of approximately 3 hours. The organic layer was extracted with ethyl acetate and concentrated for injection onto the chiral column. This would not only reveal the selectivity of the enzymes but would also permit us to identify the optimal time for terminating the reactions with respect to conversion and selectivity.

Finding a method that enabled separation of the compounds from the enzyme reactions on the C-18 column by HPLC was straightforward. However, methods developed using C-18 columns are not transferable to chiral columns and new methods needed to be developed. We evaluated separation of our compounds with four different chiral columns, namely the Chiralcel OJ, Lux 5 μ Cellulose-1, Chiralpak AD-H and Lux 5 μ m Amylose-2 columns. We proposed to find a method that would ideally separate both the substrate and hydrolysis standard enantiomers, thus displaying four distinct peaks in the chromatogram. Once the standard run provided sufficient separation, we could subject the samples from the enzymatic reactions for analysis.

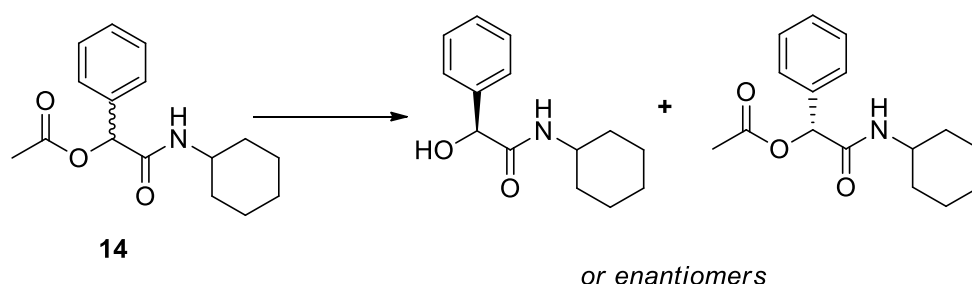
We began first by examining the OJ column. A standard sample containing both the Passerini product (**12** – **14**) with its corresponding hydrolysed compound (**15** – **17**) was dissolved in acetonitrile and injected onto the column. The mobile phase consisted of an IPA/ hexane solution as these are the

solvents permitted for use on this column. The first approach we used was an isocratic elution of IPA/ hexane mixtures, with ratios varying between 1:99 to 99:1. This afforded no separation for any of the compounds. The elution times for selected solvent ratios were over 60 minutes and only one large distorted peak would appear. We shifted to a gradient elution and still no baseline separation was seen. We concluded that the OJ column was not suitable for the compounds of interest and hence resorted to another option.

The second column employed was the Cellulose-1 chiral column. For this the same approach was applied as for the previous column using various proportions of IPA/ hexane solutions. This too afforded no baseline separation for any of the three Passerini compounds and hydrolysis adducts.

Subsequently we tested the AD-H column. A method was established that resulted in partial separation of **12/15** and **13/16**. This column produced the best chromatogram for **14/17** and these results will be discussed in **2.2.4.1**.

2.2.4.1 Enantioselectivity of the EKR of **14**



Scheme 29: Proposed EKR of the Passerini compound **14**. Reagents and conditions: Lipase 6Y/6Z, 50% MeCN in 50 mM phosphate buffer, pH 7.0, 40 °C, 3 h at 20 min intervals.

The method that delivered optimal separation of **14/17** on the AD-H chiral column consisted of a 6:94 IPA/ hexane mobile phase. Using an isocratic elution, the run persisted for ~35 minutes. Although this was time-consuming, it was the only method that afforded a pleasing result. Figure 15 shows the chromatogram obtained from the standard injection of **14** and **17**. Four peaks were observed. The Passerini enantiomers eluted at ~17- and ~26 minutes. The hydrolysed standard **17** had elution times of ~14- and ~34 minutes.

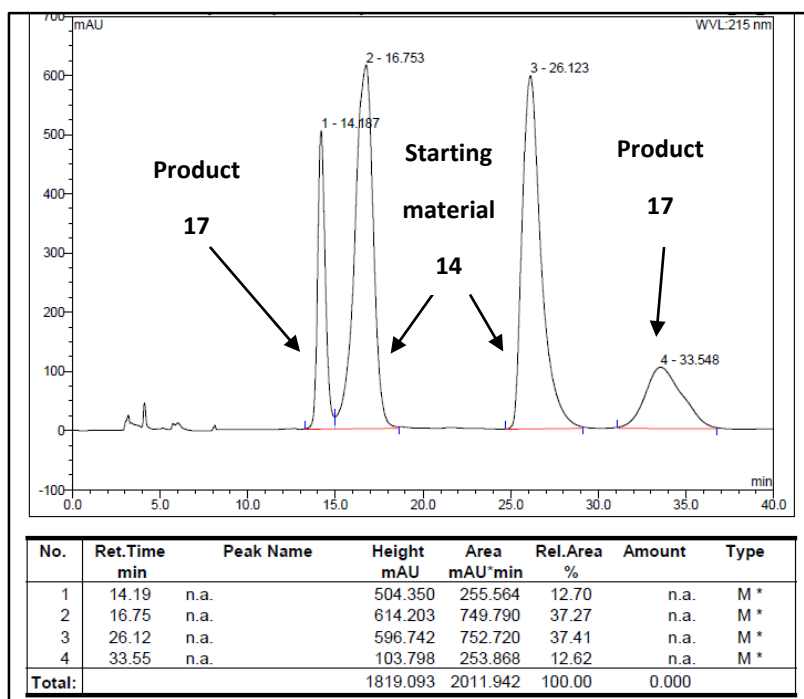


Figure 15: Standard injection of **14** and **17** on the AD-H chiral column (6:94 IPA/ hexane).

The relative peak areas reflect equal amounts for each of the set of enantiomers as expected for racemates - ~37% for both enantiomers of **14** and ~13% for **17** (Figure 15). With separation of the standard sample having been achieved, we were now primed to subject the EKR reaction mixtures to the chiral column. The control reaction only displayed the substrate **14** at ~17- and ~26 minutes (in a 50:50 ratio), establishing that no reaction occurred in the absence of the lipases. The first EKR injection was performed after 20 minutes. We began initially with assessing the reactions from Lipase 6Y (Figure 16). At this time, the enzyme had hydrolysed some of the substrate to the product. The relative peak area of one substrate enantiomer decreased from 50% to 44.06% and the other decreased from 50% to 48.51% (Figure 16). The hydrolysed product enantiomers which were not present at the start of the reaction, were now increased to 5.82% for one and 1.60% for the other one. Thus one of the enantiomers of the hydrolysed product which eluted at 13.80 minutes was greater than the other at 33.89 minutes. This shows that Lipase 6Y is somewhat selective as it reacted with one of the enantiomers at a faster rate than the other.

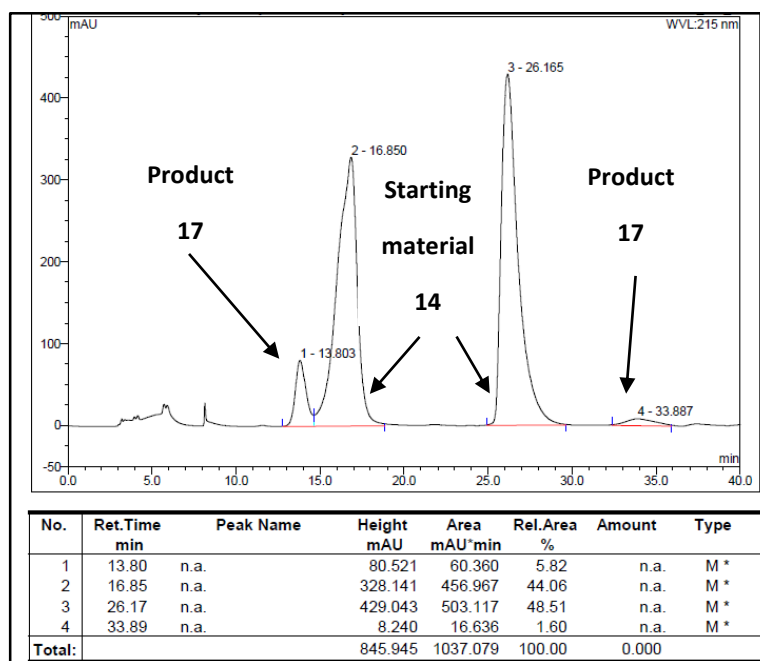


Figure 16: EKR of **14** with Lipase 6Y at 20 minutes.

The reaction was followed after every 20 minutes for Lipase 6Y and at each time-period, both of the substrate **14** peaks showed a decrease in peak area. The product peaks also increased at each phase, with the product that elutes at ~14 minutes forming more rapidly. Figure 17 depicts the final injection of the Lipase 6Y reaction with **14**. This was obtained 160 minutes after the onset of the reaction. The chromatogram reveals that the enzyme hydrolysed the one enantiomer at 16.88 minutes much faster to generate the corresponding hydrolysed product at 13.84 minutes. The lipase reacted almost completely with the said substrate, thus Figure 17 shows that only roughly a ratio of 1:13 of that enantiomer remained at this point. The other enantiomer at 26.03 minutes was not hydrolysed to the same extent as 13 times the amount of it still remained unreacted at 160 minutes as compared to the opposite enantiomer. It was apparent that Lipase 6Y preferred the one enantiomer above the other, but still hydrolysed a fraction of the other, making separation by EKR not as effective as desired.

Following the same procedure with the reactions of Lipase 6Z, we noticed overall similar results, but the reaction occurred at a swifter pace. From Figure 18, it is evident that the enzyme is hydrolysing one of the two enantiomers of the substrate quicker than the other. At merely 20 minutes, more than half the amount of substrate at 16.60 minutes was already hydrolysed to the product at 13.89 minutes. This contrasts with Lipase 6Y as approximately five times less the amount of the same hydrolysed

product was generated at 20 minutes (Figures 16 and 18). In comparison, a ratio of approximately 1:4 of the other hydrolysed enantiomer was also formed and eluted at 33.67 minutes (Figure 18).

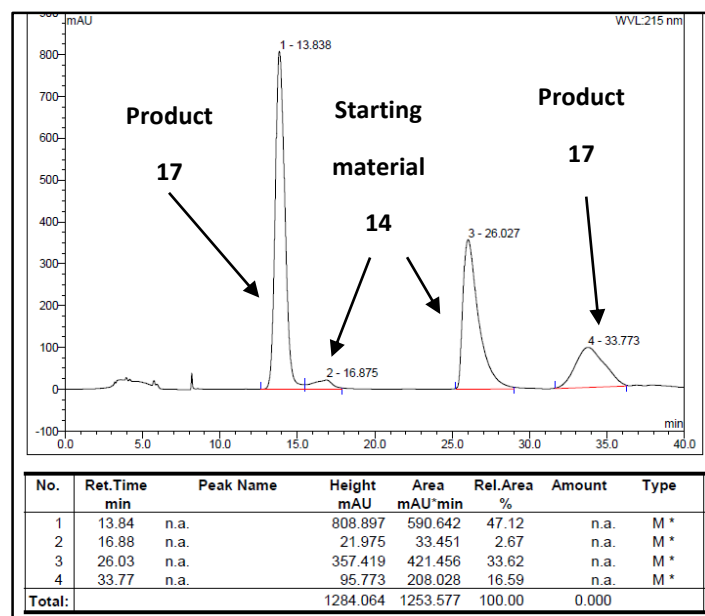


Figure 17: EKR of **14** with Lipase 6Y at 160 minutes.

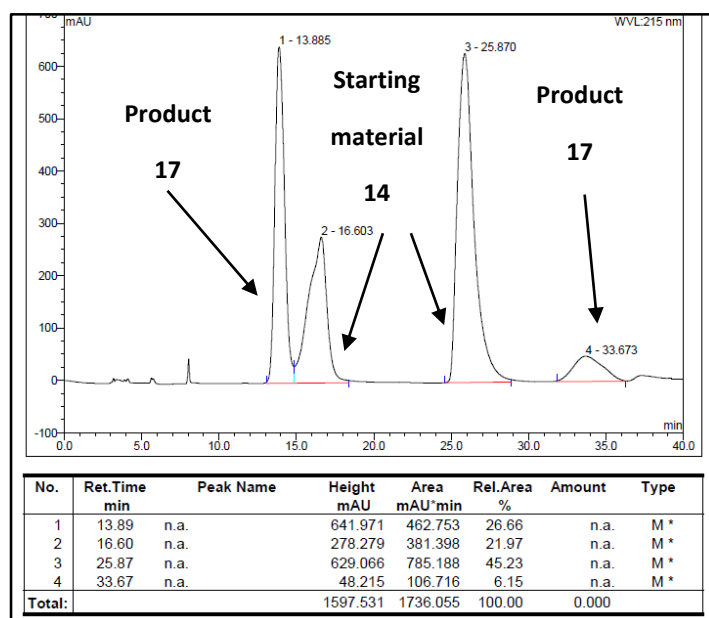


Figure 18: EKR of **14** with Lipase 6Z at 20 minutes.

Akin to Lipase 6Y, Lipase 6Z reactions also showed escalating amounts of the hydrolysis products every 20 minutes, more so with the enantiomer at ~14 minutes. The substrate peaks also reduced congruently. The last chromatogram acquired from the set of Lipase 6Z reactions was at 120 minutes (Figure 19). These results were consistent with those of Lipase 6Y. Only roughly a ratio of 1:11 of the preferred substrate remained and three times the amount of the complementary hydrolysed product was produced as compared to the other product enantiomer. Lipase 6Z produced three times less the amount of the hydrolysed product (at 33.86 minutes) in comparison to the other enantiomeric product, and hence approximately 11:1 of the less preferred substrate was still present after 2 hours.

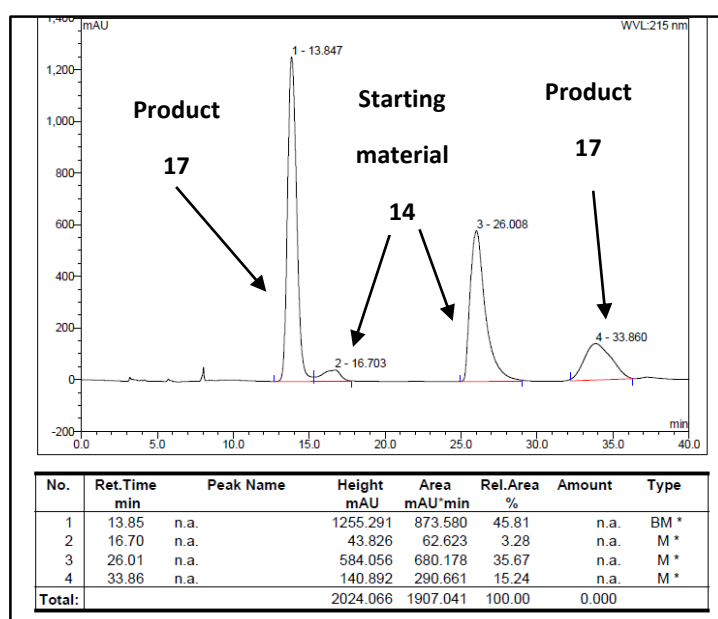


Figure 19: EKR of **14** with Lipase 6Z at 120 minutes.

The outcomes of Lipase 6Y and 6Z were almost equivalent with the exception that Lipase 6Z initially reacted with the Passerini compounds quicker. However, both biocatalysts showed some selectivity as one enantiomer was hydrolysed quicker than the other from the commencement of the investigation. Using the relative peak areas achieved from the AD-H chiral column, we calculated the conversion and e.e. at each time interval to gain statistical insight into the EKR of **14**.

An example of the calculation of the e.e. of both the substrate (ee_s) and product (ee_p) of the EKR of **14** is contained within the Appendix. The conversion (c) of the enzymatic reaction was determined using Equation 1. In 1982 C.J. Sih founded a dimensionless parameter to express the enantioselectivity (E)

of an EKR reaction.⁸⁶ This value is meant to remain constant during the course of the reaction and is only dependent on the nature of the process. E is related to both the conversion and e.e. for irreversible ester hydrolysis such as the EKR studies of the Passerini compounds in this project, and can be computed using either Equation 2 or 3.^{86, 87} These parameters have been calculated using the aforementioned equations for the various time phases for the EKR of **14** with Lipases 6Y and 6Z and these are listed in Tables 5 and 6.

$$\text{Conversion} = ee_s / (ee_s + ee_p) \quad \dots \text{Equation 1}$$

$$E = \ln[1-c(1+ee_p)] / \ln[1-c(1-ee_p)] \quad \dots \text{Equation 2}$$

$$E = \ln[(1-c)(1-ee_s)] / \ln[(1-c)(1+ee_s)] \quad \dots \text{Equation 3}$$

Table 5: Enantioselectivity parameters of **14** with Lipase 6Y.

Time (min)	ee _p (%)	ee _s (%)	conversion (%)	E
20	56.8	4.80	7.79	3.80
40	56.3	9.64	14.7	3.93
60	65.6	13.9	17.5	5.51
80	68.9	17.9	20.6	6.46
100	68.2	23.0	25.2	6.60
120	62.9	45.4	41.9	6.79
140	61.5	52.6	46.1	6.99
160	47.9	85.3	64.0	7.24

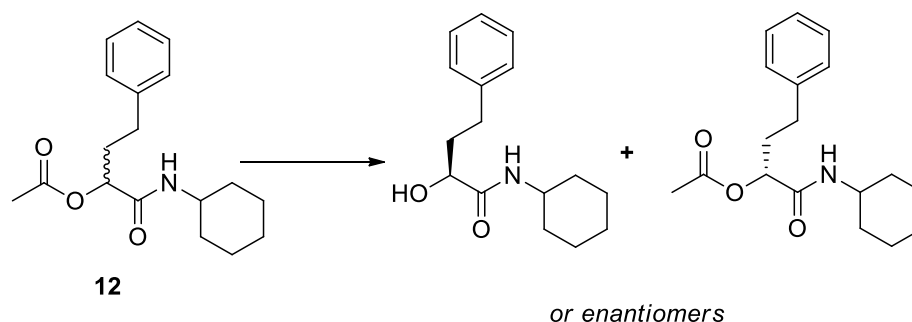
Table 6: Enantioselectivity parameters of **14** with Lipase 6Z.

Time (min)	ee _p (%)	ee _s (%)	conversion (%)	E
20	62.5	34.6	35.6	6.04

40	50.8	32.9	39.3	4.18
60	62.8	47.3	43.0	6.90
80	56.0	66.2	54.2	6.85
100	53.8	78.5	59.4	7.60
120	50.1	83.2	62.4	7.39

From the numerical data, it is apparent that the enzymatic reactions were only somewhat enantioselective. The enantiomeric excess values (ee_s and ee_p) for Lipase 6Y and 6Z (Table 5 and 6) were not high enough to allow practical separation of the enantiomers. A general decrease for ee_p was observed as the reactions progressed for both enzymes, whereas ee_s displayed an increase over time as would be expected. Customarily an enantioselectivity or E value of 15 to 30 is considered as average to good selectivity. Any result below or exceeding this is interpreted as poor or exceptional correspondingly.⁸⁸ The E- values obtained for the EKR of **14** are low, with no result greater than 8 for both lipases at any given time. What is noticeable from Table 5 is that E- values obtained at very low conversions are not reliable, and stabilise to a steady number once conversions are higher.⁸⁸ This proves that whilst the enzymes successfully hydrolyse the Passerini enantiomer **14**, they do so with only partial selectivity and are hence undesirable for functional purposes. The HPLC information reflects that the lipases are slightly discriminative towards the enantiomers, but the enantioselectivity ratios provide absolute values that give a good indication of how practically useful the EKR is likely to be.

2.2.4.2 Enantioselectivity of the EKR of **12**



Scheme 30: Proposed EKR of the Passerini compound **12**. Reagents and conditions: Lipase 6Y/6Z, 50% MeCN in 50 mM phosphate buffer, pH 7.0, 40 °C, 3 h at 20 min intervals.

We also attempted separation of **12/15** on the chiral AD-H column as it worked proficiently with **14/17**. A mobile phase of 9:91 IPA/ hexane was the only method that achieved 4 peaks corresponding to both sets of enantiomers. The elution time was quicker than with **14/17** and only lasted 16 minutes for the standard run. Unfortunately, a fifth peak was also observed which interfered with the baseline separation as it co-eluted with one of the substrate and hydrolysis product peaks. Hence accurate peak area quantities could not be obtained. We presumed that the additional peak was only present in the standard injection, thus we tested the enzymatic reactions using this same method on the AD-H column.

The EKR reactions were performed under the conditions stated in Scheme 30. As done previously, a portion of the reaction was extracted at 20 minute intervals, concentrated and injected onto the column. Unfortunately, with the enzymatic reactions, the additional peak still appeared in the chromatogram and interposed on the separation of the compounds of interest. We set to determine what the added peak was due to, but it was unidentifiable by any means.

Owing to the constraints from the unknown contaminant spotted on the AD-H column, we shifted to an Amylose-2 chiral column. With this we managed to separate only the hydrolysed enantiomer **15**, with one peak only seen for **12**, totalling 3 peaks for the standard run (Figure 20). Although separation of both pairs of enantiomers is ideal, we could still acquire the enantioselectivity parameters from 3 peaks. The solvent system used for this was 10:90 IPA/ hexane. Figure 20 depicts the Passerini compound at 29.39 minutes and the hydrolysis standards at 9.93- and 18.37 minutes, both roughly at equal peak area percentages as predicted.

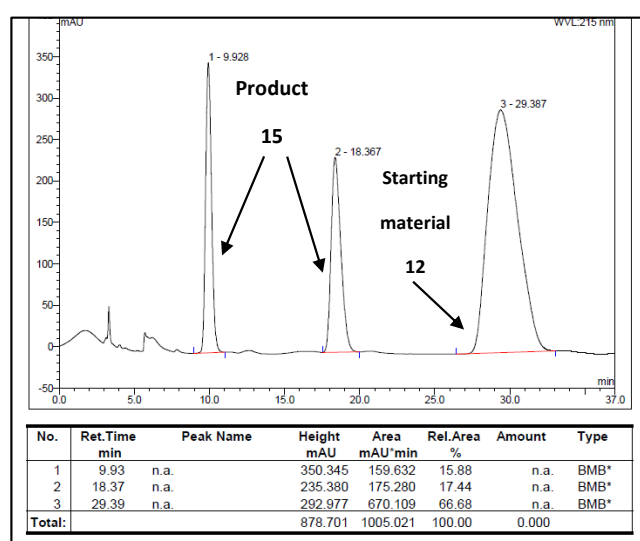


Figure 20: Standard injection of **12** and **15** on the Amylose-2 chiral column (10:90 IPA/ hexane).

With a successful method in hand, we were now in a position to examine the EKR of the Lipase 6Y reactions. At 20 minutes, the enzyme had already hydrolysed both of the substrate enantiomers to some extent. A hydrolysis product peak was noted at 9.92 minutes in a relative quantity of 5.07%. Another peak for the other hydrolysed enantiomer at 18.54 minutes was seen at 36.05%. From this it was already evident that the lipase was fairly selective as one hydrolysed compound was formed more rapidly. This pattern continued over the course of the reaction until the 60-minute mark, where unusual results were obtained. Figure 21 shows the substrate peak at 28.04 minutes reduced to a relative amount of 36.66% and one hydrolysed enantiomer at 18.44 minutes increased to 58.65%. The disadvantage of the practice of EKR is that any of the two enantiomers can only be obtained at a maximum yield of 50%, given that the corresponding substrate enantiomer is completely consumed in the reaction. From Figure 21, we noticed that the hydrolysed enantiomer at ~18 minutes already exceeded 50% at 60 minutes.

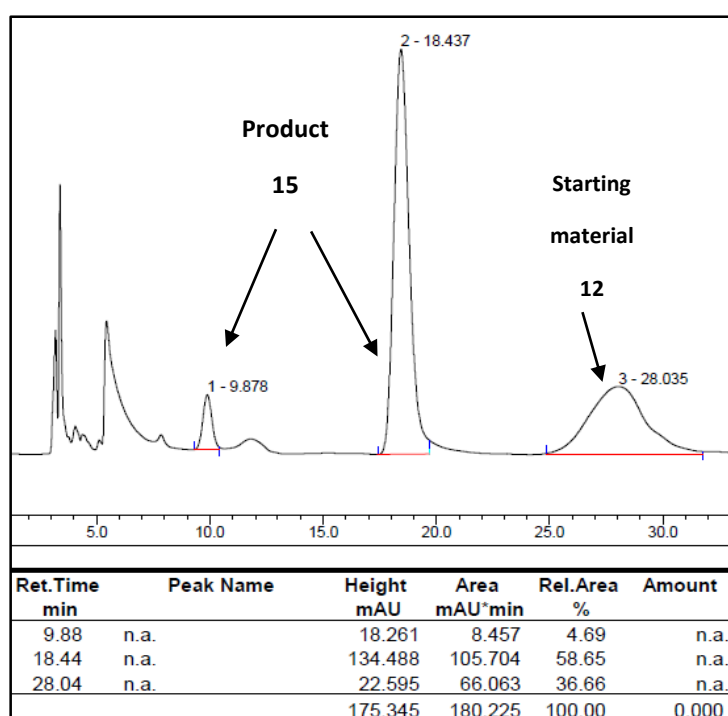


Figure 21: EKR of **12** with Lipase 6Y at 60 minutes.

We resumed the reaction up till 180 minutes and injected the samples onto the chiral column. The hydrolysed product at 17.54 minutes increased even further to 84.93% (Figure 22). The substrate peak

at 28.96 minutes almost entirely disappeared with only 3.09% remaining. These results were unreasonable as even if the enzymes were not selective and hydrolysed both the substrate enantiomers at the same rate, 50% of each of the hydrolysed products would be seen. Figure 22 shows only 11.98% of the other enantiomer at 9.48 minutes. It is most obvious from Figure 22, with almost all of the starting material hydrolysed, there still seemed to be significant selectivity, which is not possible. The only justification was that another unknown side-reaction was perhaps occurring that would give rise to a product that elutes at the same position as the favoured hydrolysed product (~18 minutes), causing the relative peak area to surpass the 50% maximum yield from EKR.

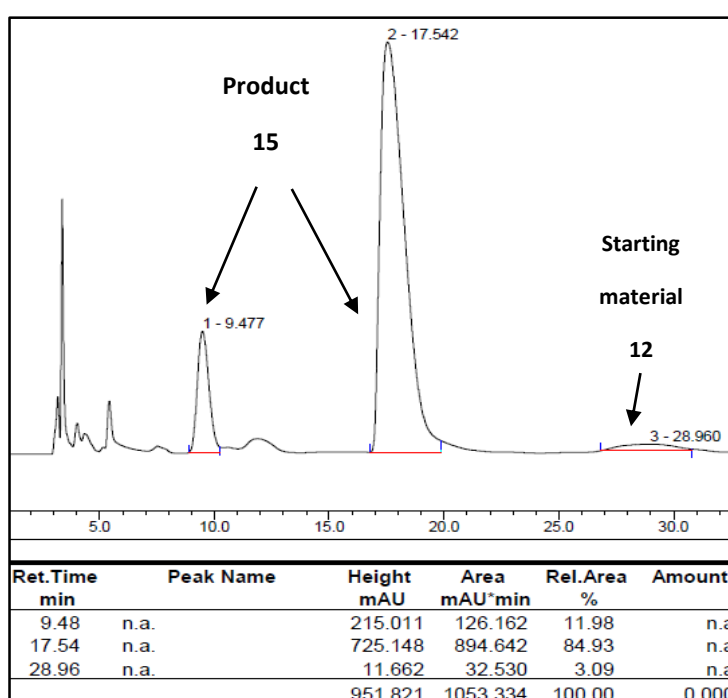


Figure 22: EKR of **12** with Lipase 6Y at 180 minutes.

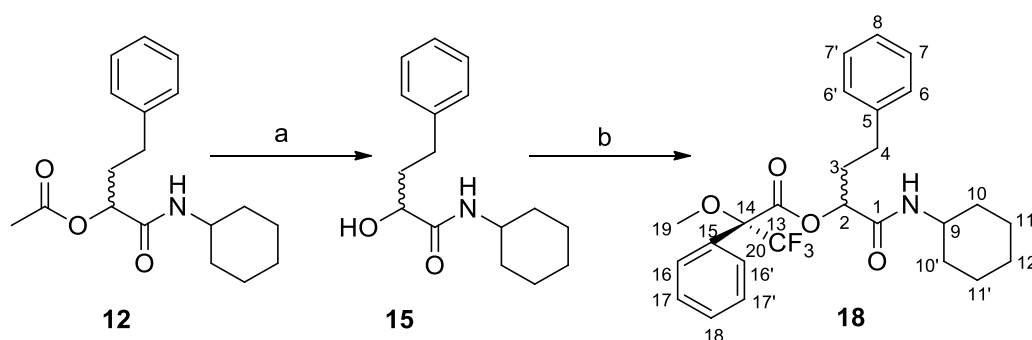
We subjected the enzymatic reactions from Lipase 6Z at each time interval onto the column. Likewise, the same irrational outcomes were observed from 100 minutes where the hydrolysed product was yielded over 50% and the starting material was almost completely reacted, even though the enzyme exhibited notable selectivity. At 180 minutes, only 21.6% of the substrate remained unreacted, and the favoured hydrolysed enantiomer was present at 71.26%.

The AD-H and Amylose-2 chiral columns yielded data that was inexplicable for the Passerini compound **12**. Although we could establish that the enzymes were hydrolysing the racemic mixture in a somewhat selective manner, we were unable to determine the enantioselectivity of the reactions with both Lipase 6Y and 6Z as the relative peak area values were inaccurate. This led us to a new solution to verify the degree of selectivity of the lipases during EKR of **12**.

In 1969, Mosher described an NMR-focussed technique termed the Mosher ester analysis, used to infer the absolute configuration of an otherwise unidentified chiral secondary alcohol centre.⁸⁹ The mechanism involves transforming an enantiomeric mixture into a diastereomeric mixture (Mosher's esters) by coupling it to a reagent that is itself chiral (Mosher's acid of known chirality). The ¹H NMR spectra of enantiomers are indistinguishable, but once the Mosher's esters are formed, the diastereomeric protons will display different chemical shifts. By evaluating the variance in the chemical shifts of resonances of analogous protons of the two diastereomers, the absolute stereochemistry of the original enantiomers can be deduced.⁸⁹

Another function of the Mosher ester analysis, which is of interest in this project is the inference of the proportion, or e.e. of enantiomers present in a certain sample. This is accomplished in the same way used to determine absolute configuration. Mosher's ester diastereomers must be prepared, and from either the ¹H or ¹⁹F NMR spectra, the integration of corresponding protons or fluorine atoms from the two compounds are measured, offering a direct correlation to the concentration of each of the original enantiomers present in the mixture.⁸⁹

With the complications experienced from the chiral HPLC columns with **12**, we used the Mosher ester method to measure the e.e. for the EKR of **12** with Lipases 6Y and 6Z.



Scheme 31: Mosher ester analysis of the EKR of **12**. Reagents and conditions: a) Lipase 6Y/6Z, 50% MeCN in 50 mM phosphate buffer, pH 7.0, 40 °C, 12 h b) (*R*)-MTPA, DCC, DMAP, CH₂Cl₂, 90 min.

The analysis was completed in two steps as demonstrated in Scheme 31. First a large-scale EKR reaction was performed (200 mg of **12**) under the same conditions as stated previously. However the reaction proceeded for 12 hours and was not evaluated at 20 minute intervals as with those reactions subjected to chiral chromatography. The organic layer was then extracted with ethyl acetate and concentrated. Since Lipases 6Y and 6Z did not hydrolyse the substrate completely, the residues contained both the starting material **12**, and the hydrolysed compound **15** as observed from TLC. Hence prior to carrying out the second step (b) of the analysis, the mixture was purified by column chromatography to obtain pure **15**, as a scalemic mixture from both the enzyme reactions. A yield of 22% of **15** was achieved from the Lipase 6Y reaction and 26% from Lipase 6Z, denoting that the enzymes hydrolysed the substrate at a very similar rate. Since a large amount of starting material was recovered from the column, it was expected that the yields would be notably low.

In addition, the crude residues from the enzyme reactions were subjected to HPLC on the C-18 column to determine the conversion from EKR. The method used previously for separation of **12/15** on the C-18 column was applied. With this, the hydrolysed enantiomeric products eluted at 4.02 minutes and the substrate mixture at 4.77 minutes. Figure 23 indicates that 45.7% of **15** was yielded in 12 hours by Lipase 6Y, whilst 31.2% was the yield by Lipase 6Z as displayed in Figure 24. The conversion values obtained from this reactions on the C-18 column are regarded as semi-quantitative data as they are based only on peak area and not standard curves.

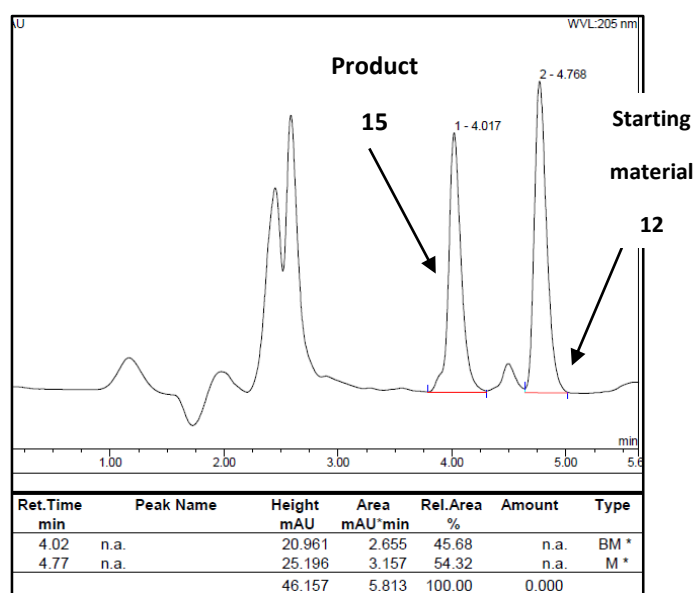


Figure 23: EKR of **12** with Lipase 6Y at 12 hours.

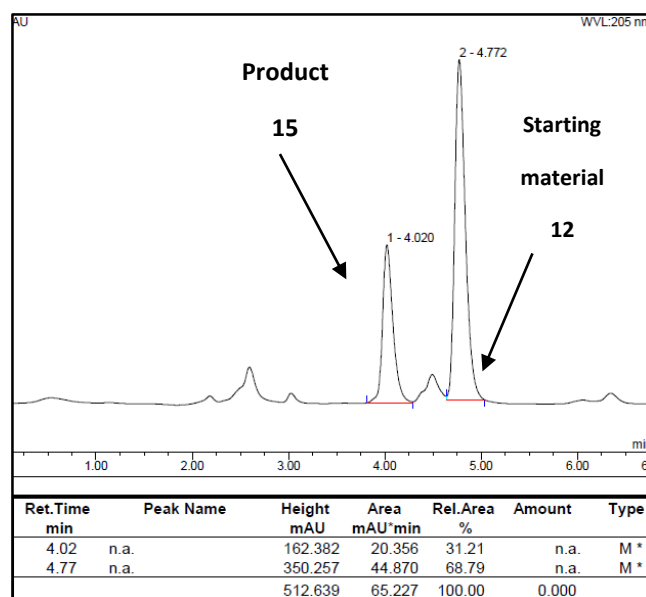


Figure 24: EKR of **12** with Lipase 6Z at 12 hours.

With the scalemic mixtures of **15** from the lipase reactions in hand, we proceeded with the synthesis of **18** from the Mosher reaction as shown in Scheme 31 (step b). The scalemic mixture of compound **15** was dissolved in CH_2Cl_2 and the Mosher's acid (*R*)-MTPA, DCC and DMAP were added to the solution in stages. After 90 minutes of stirring, the product was extracted and isolated as a mixture of two diastereomers by column chromatography in a moderate yield of 67% from the Lipase 6Y reaction. The Lipase 6Z material yielded **18** at 78%.

Successful preparation of **18** was substantiated spectroscopically. Infrared analysis exhibited two stretches for the amide and newly formed ester carbonyl groups of **18** at 1660- and 1753 cm^{-1} , respectively. Significant stretches for the NH (3306 cm^{-1}) and C-F (1017 cm^{-1}) moieties were also noted.

The ^1H NMR spectra of **18** from Lipase 6Y and 6Z reactions are in essence identical, and confirm the effective synthesis of the Mosher's esters. Two multiplet signals were apparent at 7.63 – 7.01 ppm which integrated for 10 protons from the two phenyl rings. A singlet was observed at 3.55 ppm corresponding to H-19 of the methoxy group. Another vital signal was that of H-2 at the more central stereogenic centre, which appeared as a multiplet at 5.41 – 5.26 ppm. Due to probable impurities, the cyclohexyl region at 1.87 – 0.95 ppm contains 17H instead of the anticipated 10 protons.

Likewise the ^{13}C NMR spectra provided further proof of the preparation of **18** as a mixture of two diastereomers, as doubling of some peaks were noticed. Two peaks appeared in the carbonyl region

consistent with the ester and amide functionalities. A characteristic CF_3 peak was observed at 125.30 ppm as anticipated. Duplicate peaks were seen at $\sim 84 - 85$ ppm for the stereogenic centre C-14, and a single peak for the second stereogenic centre C-2 at ~ 75.3 ppm. C-19 gave rise to a peak at ~ 55.4 ppm.

To reach the desired outcomes from the Mosher ester analysis, the data revealed by the ^{19}F NMR was imperative. For both the Lipase 6Y and 6Z reactions separately, two singlet signals were seen at -70.66 and -70.82 ppm for the CF_3 groups of the two diastereomers. From the integration of the Lipase 6Y spectrum (Figure 25) the e.r. of the compounds was determined to be 1:9 which corresponds to an e.e. value of 80%. Lipase 6Z showed greater selectivity as the e.r. from the ^{19}F NMR spectrum was 1:10. Therefore, the e.e. of the product from the Lipase 6Z reaction was 90%.

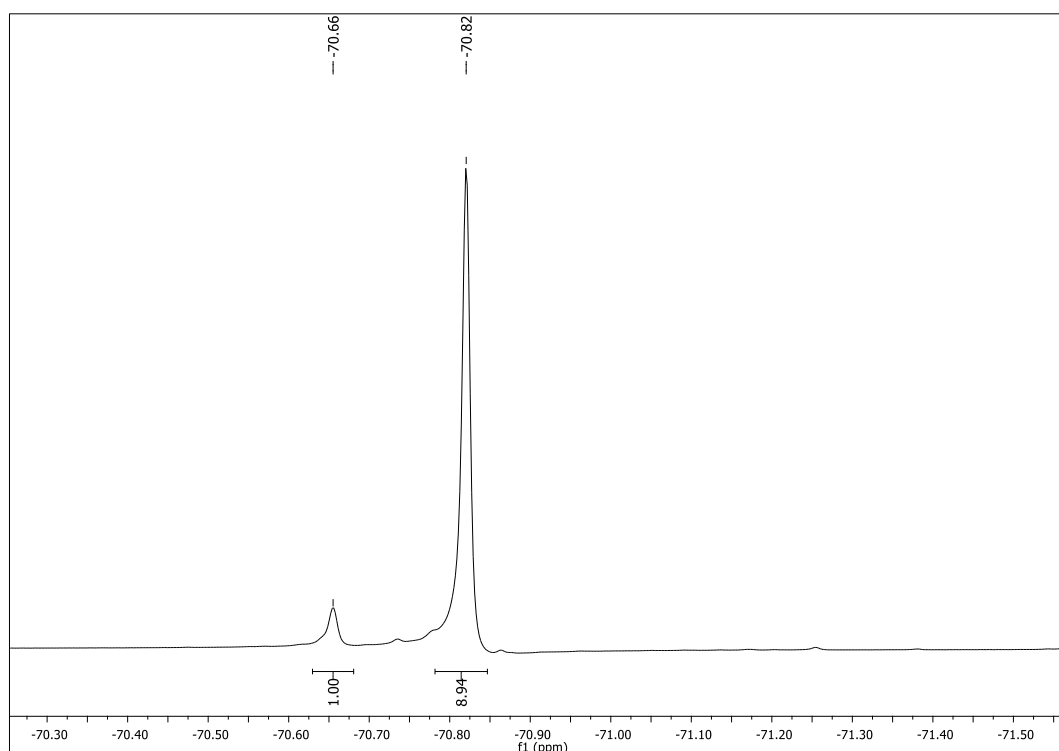
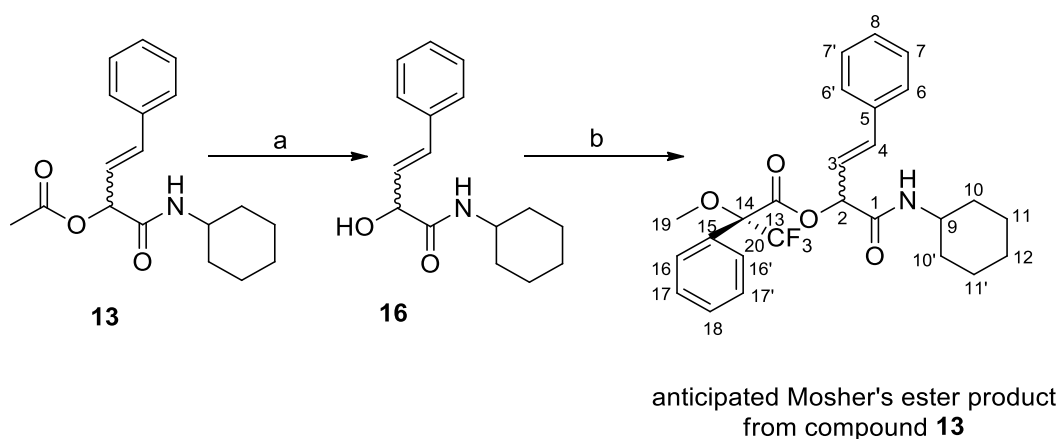


Figure 25: ^{19}F NMR spectrum of **18** obtained from the Lipase 6Y reaction.

Having obtained the semi-quantitative conversion of the Lipase 6Y and 6Z reactions of **12** from the C-18 column, and the e.e. of the hydrolysed products from the ^{19}F NMR, we calculated the E value using Equation 2. The E-value of the EKR from Lipase 6Y was found to be 14.7, and 21.7 for Lipase 6Z.

The results reveal that Lipase 6Z exhibited a greater e.e. and hence enantioselectivity towards **12** as compared to Lipase 6Y, although the latter displayed greater conversion after 12 hours. As abovementioned, E-values in the range of 15 – 30 are beneficial for practical purposes.⁸⁸ Therefore, the EKR of the Passerini compound **12** by both Lipases was successful as the E-values lie within a reasonable region. Separation of the enantiomers of **12** by Lipase 6Z would, however, be more feasible since the E-value is much greater. The enantioselectivity parameter of the EKR of **14** steadied at ~7 (Tables 5 and 6). Hence this study suggests that Lipases 6Y and 6Z react more efficiently and selectively towards **12**, making successful separation of this racemic mixture by means of enzymes more probable.

2.2.4.3 Enantioselectivity of the EKR of **13**



Scheme 32: Mosher ester analysis of the EKR of **13**. Reagents and conditions: a) Lipase 6Y/6Z, 50% MeCN in 50 mM phosphate buffer, pH 7.0, 40 °C, 20 h b) (R)-MTPA, DCC, DMAP, CH₂Cl₂, 90 min.

As mentioned in 2.2.4, separation of the **13/16** mixture using the OJ, Cellulose-1 and AD-H columns were unproductive. We resorted to the Amylose-2 chiral column and with a mobile phase of 15.5:84.5 IPA/ hexane, we managed to achieve separation of the hydrolysis products. With a standard chromatogram of 3 peaks (2 peaks for the product and one corresponding to the substrate **13**), we could gain sufficient information to determine the enantioselectivity of the Lipase 6Y and 6Z reactions.

We continued analysing the enzymatic reactions on the column at 20 minute intervals as done with the preceding compounds. Unfortunately, the substrate and one of the hydrolysed enantiomers co-eluted at each of the injections, disturbing the baseline separation we had achieved in the standard

run. As we could not obtain the necessary data from the HPLC analysis, we opted to carry out the Mosher ester analysis of **13** as it produced successful results with **12**.

In the same way as for the Passerini compound **12**, a large-scale enzymatic reaction of **13** was performed according to step *a* in Scheme 32. The reaction was left to stir for 20 hours, which was much longer in comparison to the EKR of **12**. The lipases appear to react much slower with compound **13** than with the other Passerini analogues. This was noted even in **2.2.3** (Figures 11 and 12) where the rate of hydrolysis of **13** by Lipases 6Y and 6Z was less than half that of the other Passerini enantiomers within the same time-period.

The lipases did not hydrolyse the racemic mixture of the substrate entirely, hence column chromatography was carried out to separate the starting material from the scalemic mixture of **16**. Pure **16** was recovered in an unsatisfactory yield of 14% from EKR of Lipase 6Y. A slightly improved yield of 48% was achieved by the Lipase 6Z reaction. To deduce the conversion, the method applied in **2.2.3** for the separation of **13/16** on the C-18 column was utilised, eluting the substrate at 4.51 minutes and the hydrolysed adduct at 3.91 minutes. From HPLC, the yield of the product **16** was 21% due to Lipase 6Y (Figure 26). Figure 27 depicts a comparable yield by Lipase 6Z at 24%.

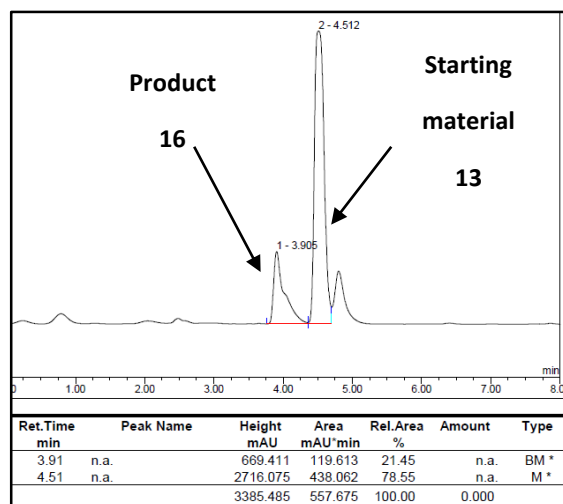


Figure 26: EKR of **13** with Lipase 6Y at 20 hours.

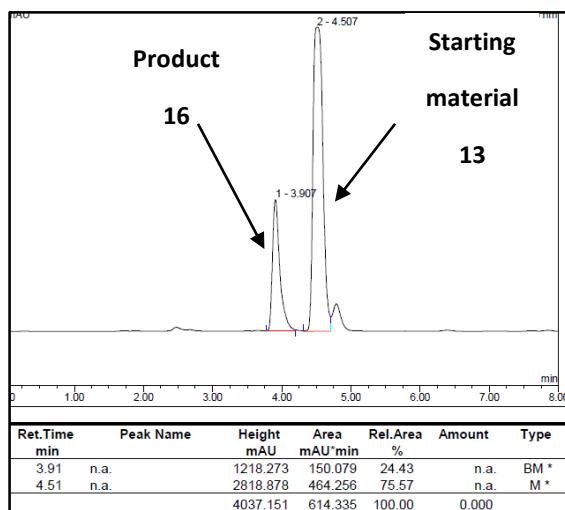


Figure 27: EKR of **13** with Lipase 6Z at 20 hours.

Although the conversion of the Passerini compound by the lipases afforded a minimal quantity of **16**, we were still able to conduct the Mosher's reaction (step *b* of Scheme 32). We undertook the synthesis in the same manner described for compound **12**. After 90 minutes, TLC indicated a new spot and total consumption of the starting material. Following extraction and purification, the liquid product obtained was characterised.

From assessment of the ^1H and ^{13}C NMR, we reached the conclusion that the expected Mosher's ester products were not effectively prepared, either from the Lipase 6Y or the Lipase 6Z reaction. The spectra offered no indication of starting material or the Mosher's acid reagent. We were not able to determine what compound had in fact been formed. The ^{19}F NMR showed 2 peaks of roughly equal integration, which meant that the product formed contained fluorine atoms. However, we could not ascertain the enantioselectivity parameters from this result. We further analysed the compounds by mass spectroscopy, and no proof of the desired product was obtained.

Evidently, the Passerini compound **13** was seemingly problematic from the onset. In 2.2.2 we attempted to chemically hydrolyse **13** under basic conditions as it yielded successful outcomes for **12** and **14**. However this was the first complication as it met with failure, and another route to preparing the hydrolysis standard **16** had to be taken. From the screening of the enzymatic reactions, **13** had the lowest conversion compared to its counterparts. Granted that the enzymes responded to this compound modestly, the enantioselectivity of the reactions could nonetheless not be elucidated by HPLC nor the Mosher ester analysis.

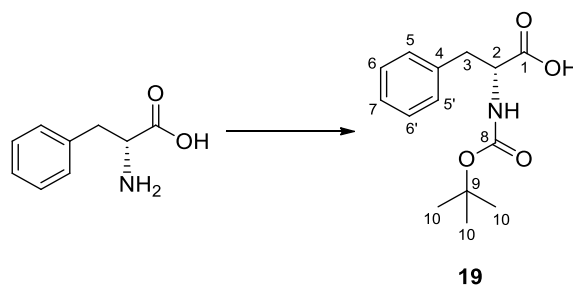
Having had no fruitful outcomes with the proteases on the Ugi compounds, we were pleased with the lipase response to the Passerini enantiomers. Although no inferences could be made regarding compound **13**, we discovered that the lipases were not very selective towards **14**. A positive result was obtained with the substrate **12**, as not only does the enzymes hydrolyse the ester functionality, it also displays adequate enantioselectivity making separation of this racemic mixture rather promising. Due to time constraints, separation of this compound was not possible, but will be considered for future work.

2.3 Diastereoselectivity Studies of the Passerini Reaction

The following task we pursued involved diastereoselectivity studies of the Passerini reaction. This work is a progression based on that executed by Zimuwandeyi.⁸⁰ Recent studies by Zimuwandeyi in our laboratories have shown that the use of chiral starting materials in the Passerini reaction can have a noteworthy impact on the reaction diastereoselectivity. Using *N*-Boc-phenylalaninal (which contains a fixed stereogenic centre) with achiral isocyanides and carboxylic acids resulted in a d.r. of 2:1 in most cases. When employing a chiral isocyanide and aldehyde in the Passerini reaction, the d.r. reduced significantly.⁸⁰ Henceforward we intended to further explore the diastereoselectivity of the reaction by introducing a combination of starting material with fixed chirality into the Passerini reaction.

2.3.1 Preparation of *N*-Boc-D-phenylalanine **19**

The first step was the synthesis of the starting material. As most HIV PIs contain a phenylalanine moiety (including the potential HIV PI compounds synthesised by Zimuwandeyi)⁸⁰, the majority of the reagents we prepared were based on *N*-Boc-phenylalanine. For the acid component in the Passerini reaction, we utilised both the natural and unnatural amino acid derivatives, *N*-Boc-L-phenylalanine and *N*-Boc-D-phenylalanine, respectively. The former was obtained commercially hence we were only required to prepare the unnatural analogue via Boc-protection of D-phenylalanine (Scheme 33).



Scheme 33: Reagents and conditions: (Boc)₂O, 1,4-dioxane, 1 M aq NaOH, 24 h.

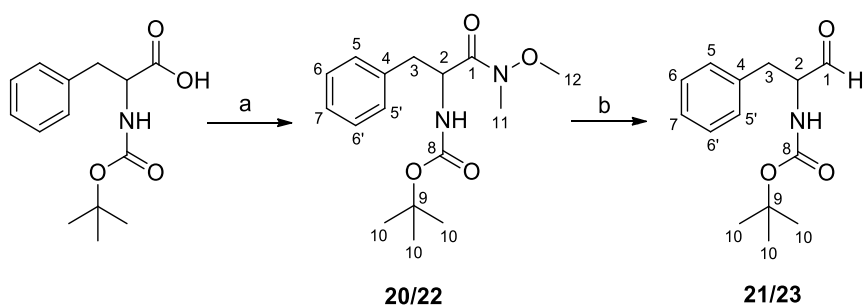
The synthetic route applied was based on the work of Majumdar *et al.*⁹⁰ D-phenylalanine was dissolved in 1,4-dioxane, followed by the addition of NaOH at 0 °C. After 20 minutes of stirring, Boc-anhydride was gradually added and the reaction continued overnight at room temperature. Following neutralisation of the mixture by KHSO₄, the desired product **19** was extracted and purified resulting in a yield of 67%.

The Boc-protection of D-phenylalanine was carried out successfully. The ¹H NMR spectrum depicts a multiplet at 1.60 – 1.08 ppm integrating for 9 protons (H-10). The H-10 protons are expected to appear as a singlet as they do not couple to any other protons, hence the multiplet observed could be due to the presence of rotamers. Only one proton is present from NH at 6.66 ppm, indicative that the nitrogen was effectively Boc-protected. A singlet OH peak was also evident at 10.96 ppm. ¹³C NMR spectroscopy shows doubling of the majority of signals. Two signals at 156.76- and 155.47 ppm which both correspond to the newly formed Boc-group are observed. The tertiary carbon atom, C-9, is also represented by two peaks at 81.65- and 80.17 ppm. The characteristic C-10 signal appears as a pair of signals at 28.27- and 27.97 ppm. Both the ¹H and ¹³C NMR data support the existence of rotamers of **19** since doubling of the signals is seen. The presence of rotamers could be the result of H-bonding between the OH proton and nitrogen, or the double-bond nature of the C-N amide bonds.

IR spectroscopy further verifies the presence of **19**. Two C=O signals are noticed at 1709- and 1644 cm⁻¹ consistent with the carboxylic acid and carbamate functionalities. A broad OH signal also occurs at 3245 cm⁻¹ and an NH peak at 3306 cm⁻¹.

2.3.2 Preparation of *N*-Boc-D- and L-phenylalaninal

We planned to use *N*-Boc-D- and L-phenylalanine as the carboxylic acid constituents in the Passerini reaction. Moreover we also utilised these acids as a starting material to prepare the aldehyde components, *N*-Boc-D-phenylalaninal and *N*-Boc-L-phenylalaninal. According to Scheme 34, the aldehydes were prepared in two steps via the Weinreb amide of phenylalanine, employing the well-known procedure established by Fehrentz *et al.*⁹¹



Scheme 34: Reagents: a) CDI, CH₂Cl₂, NH(OMe)Me.HCl b) LiAlH₄, THF.

We commenced with preparation of the Weinreb amide (Scheme 34, step *a*) of the unnatural amino acid derivative **20** from *N*-Boc-D-phenylalanine **19** (discussed in **2.3.1**). CDI was added in small amounts to a solution of compound **19** in CH₂Cl₂. Effervescence was observed at each addition due to the evolution of CO₂. After 60 minutes of stirring, *N,O*-dimethylhydroxylamine hydrochloride was added and the reaction resumed for 48 hours. Ensuing the work up procedure, TLC indicated a single product and no starting material. Hence no further purification was necessary and the expected compound was achieved in an excellent yield of 90%.

A definite indication of the formation of **20** is two singlets at 3.65- and 3.16 ppm in the ¹H NMR spectrum, due to H-12 and H-11, respectively, each integrating for 3 protons. The absence of an OH peak which was seen for **19** at 10.96 ppm also confirmed that the Weinreb amide had been formed. The Boc-group appeared as a singlet at 1.38 ppm, in contrast to the multiplet observed for **19** showing no sign of rotamers for this product. Correspondingly in the ¹³C NMR spectrum, C-11 and C-12 emerge at 32.06- and 61.54 ppm, respectively, signals which were not present in the starting material. In this spectrum only single peaks were present for each carbon environment, depicting only a single averaged conformation, unlike the rotamers obtained from **19**.

A key point from the infrared spectrum is the disappearance of the broad OH signal which was visible in the starting material at 3245 cm⁻¹.

The subsequent step involved reduction of the amide by LiAlH₄ in THF at 0 °C (Scheme 34, step *b*) to produce the *N*-Boc-D-phenylalanylaldehyde enantiomer **21** in a pleasing yield of 86%. The optical rotation of **21** was found to be $[\alpha_D]^{20}_{589} +40.6$ (c 0.50, MeOH) which is similar to the literature value, justifying that the compound had not been racemised.⁸⁰

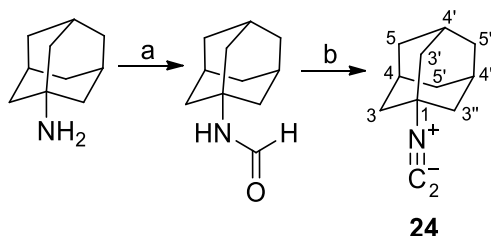
Upon inspection of the ^1H NMR data, a distinct aldehyde peak was depicted at 9.63 ppm as a singlet. The two singlet peaks due to H-11 and H-12 of compound **20** were no longer seen for **21**. ^{13}C NMR results also displayed no signals for C-11 and C-12 from the Weinreb amide. In addition, a signal at 199.37 ppm was noted, representative of the C=O of the aldehyde.

The melting point was determined to be 82 – 83 °C which is in accordance with the literature value (86 °C).⁹¹

N-Boc-L-phenylalaninal **23** was prepared efficiently in a 78% yield using the same two-step procedure as applied with **21** (Scheme 34) starting from commercially obtained *N*-Boc-L-phenylalanine. The NMR and IR spectra were fundamentally identical to those seen for **21**. The specific rotation was $[\alpha_D]^{20}_{589}$ -41.4 (c 0.50, MeOH) which is similar to that of -44.4 reported in literature and also the negative equivalent of **21**, which is predicted for enantiomers. Additionally the determined melting point of 81 – 83 °C further supports the effective synthesis of **23**.⁹¹

With the chiral carboxylic acids and aldehydes in hand, we resumed synthesis of three different isocyanides to be used in the Passerini reaction, namely adamantyl-, D-phenylalanine- and L-phenylalanine isocyanides.

2.3.3 Preparation of adamantyl isocyanide **24**



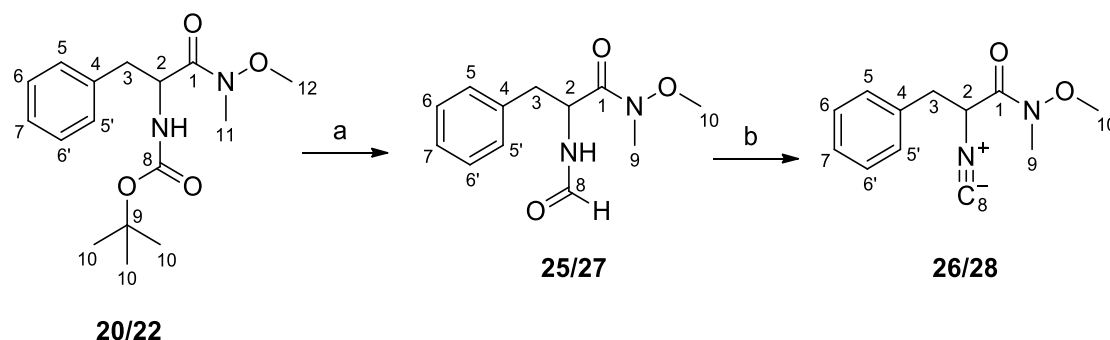
Scheme 35: Reagents and conditions: a) HCOOH , Ac_2O b) NEt_3 , POCl_3 , CH_2Cl_2 , 24 h.

Whilst adamantyl isocyanide is not chiral, Gravestock *et al.* had uncovered that branched isocyanides, like **24** led to enhanced inhibition of HIV protease.³⁴ Hence as an advancement to the endeavours of Zimuwandeyi,⁸⁰ we prepared **24** to be used in combination with chiral acids and aldehydes in the Passerini reaction to discover its effect on the reaction diastereoselectivity and compare it to that of chiral isocyanides.

As demonstrated in Scheme 35, adamantyl isocyanide was prepared via formylation (step *a*) and dehydration (step *b*) steps. Commercially purchased adamantylamine was treated with formic acid and acetic anhydride under a nitrogen atmosphere. The reaction was quenched after 24 hours to obtain crude adamantyl formamide. Without further purification, the compound underwent dehydration using triethylamine and POCl₃ in CH₂Cl₂. The reaction was then quenched using Na₂CO₃. Due to the intensity of the resulting effervescence during the work-up, a great deal of the product was lost producing lower yields. Hence it was imperative to cool the mixture to 0 °C during this step. After extraction and purification, **24** was obtained in 81% yield, as a yellow solid having a distinctive repellent isocyanide odour.

Two triplet peaks were observed in the ¹³C NMR spectrum at 151.81 ppm (C-2) and 54.21 ppm (C-1). This phenomenon, sometimes observed for isocyanides, was also encountered by Zimuwandeyi and is described further by Crews *et al.* as being attributable to the coupling of ¹³C to ¹⁴N as both are NMR-active nuclei.^{80, 92} Overall, NMR spectroscopy substantiated the synthesis of adamantyl isocyanide.

2.3.4 Preparation of D- and L-phenylalanine isocyanide



Scheme 36: Reagents: a) HCOOH, Ac₂O, TFA b) PPh₃, CCl₄, DIPEA, CH₂Cl₂.

Synthesis of the chiral D- and L-phenylalanine isocyanides was carried out in a similar fashion to **24**. Starting from the Weinreb amide of D- or L- phenylalanine (described in **2.3.2**), formylation and Boc-deprotection were achieved in a single step using the reagents stated in Scheme 36, step *a*. Only the formamide of L-phenylalanine **27** was purified and isolated in a poor yield of 28%. Crude **25** was used in the subsequent step. Both compounds were highly polar and displayed an R_f value of 0.03 in a polar mobile phase of 5% MeOH/CH₂Cl₂.

NMR proved the formation of both **25** and **27** with the singlet formamide proton peaks at 8.13- and 8.07 ppm, respectively. As expected, the Boc-group H-10 protons present in the starting material were not seen in the formamide products. Likewise, signals for C-8, C-9 and C-10 of **20** and **22** did not reflect in the desired products, but a C=O peak for the formyl group appeared at ~171 ppm for both the said compounds.

The dehydration stage was attempted using NEt₃ and POCl₃ as performed with adamantyl formamide. However, this met with failure on numerous occasions. Thus we resorted to using a combination of triphenylphosphine, carbon tetrachloride and DIPEA in CH₂Cl₂ (Scheme 36, step *b*). This route not only resulted in the desired product (17% yield for **26** and 71% for **28**), but also required no cooling and did not have problematic work-up procedures. Furthermore, no offensive smell was experienced from these chiral isocyanides.

The ¹H NMR spectra displayed no sign of the formamide proton as well as no NH peak, validating effective dehydration of **25** and **27**. No formyl C=O peak was noted in the ¹³C NMR spectra of both the isocyanide products, however representative C≡N (C-8) signals were observed for both **26** and **28** at ~159 ppm. As expected, the ¹H and ¹³C NMR spectra for the two enantiomers were identical.

The isocyanide C≡N triple bond was also reflected as a strong signal at ~2144 cm⁻¹ in the IR spectra for both **26** and **28**. No information on the optical rotation for these compounds was obtained from the literature, except the results previously obtained by Zimuwandeyi.⁸⁰ The result determined for **26** was [α_D]²⁰₅₈₉ -6.45 (c 2.00, CHCl₃). The opposite enantiomer **28** had a specific rotation of [α_D]²⁰₅₈₉ +3.55 (c 2.00, CHCl₃) which is roughly equivalent to +2.13 obtained by Zimuwandeyi for the same chiral compound under identical conditions.⁸⁰ We expect these values to be identical but opposite in sign as they are enantiomers, but they are slightly higher as compared to that of Zimuwandeyi.⁸⁰

HRMS furthermore agreed with the previous data. The calculated mass of [M + H]⁺ for the isocyanides was 219.1134, and actual masses obtained, respectively for **26** and **28** were 219.1144 and 219.1132.

Having effectually generated the chiral carboxylic acids, aldehydes and isocyanides (and the achiral adamantyl isocyanide), we were now at a stage to subject them to the Passerini reaction in various combinations to investigate their influence on the diastereoselectivity.

2.3.5 Diastereoselectivity of the adamantyl isocyanide series

We planned on synthesising three series of Passerini compounds, each employing one of the prepared isocyanides (adamantyl-, D-phenylalanine- and L-phenylalanine isocyanides) with the various chiral acids and aldehydes. The first sequence involved adamantyl isocyanide in combination with *N*-Boc-D-

or L-phenylalanine and *N*-Boc-D- or L-phenylalaninal (Figure 28). This gave rise to four different sets of diastereomeric mixtures from which the d.r. was determined. We then aimed to separate these mixtures by use of semi-preparative HPLC, which led to eight pure diastereomers for this set.

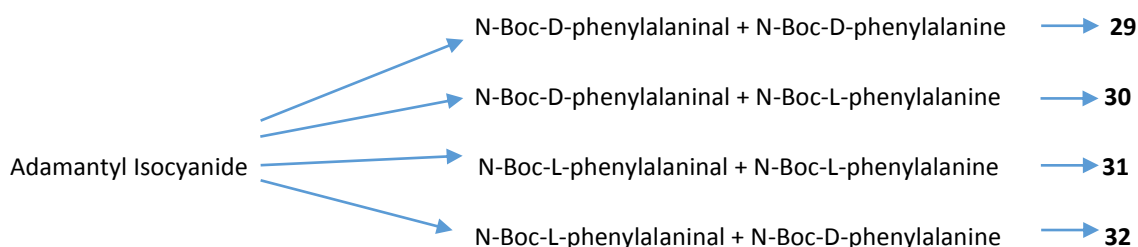


Figure 28: Synthetic plan for Passerini diastereomers using adamantyl isocyanide.

The reactions were completed using equimolar quantities of each of the starting materials in CH₂Cl₂. After 5 days of continuous stirring and purification by column chromatography, compounds **29** – **32** were achieved as a mixture of two diastereomers, in respective yields of 92, 68, 56 and 92%. Preceding separation of the mixtures, we subjected the compounds to HPLC analysis on the C-18 column for obtaining the d.r. values. These compounds, like the Passerini enantiomers in **2.2**, easily dissolved in a range of solvents. Acetonitrile was the solvent of choice for HPLC use. Baseline separation was accomplished without major challenges for all four compounds, using a gradient elution of 60:40 acetonitrile/ water to a 90:10 ratio. As expected, the enantiomers **29** and **31** (having a D/D and an L/L combination of aldehyde and acid) had elution times for both diastereomers of <20 minutes, whereas **30** and **32** (D/L and L/D mix of aldehyde and acid, respectively) which are also enantiomers eluted later at ~30 minutes. The d.r. values of this Passerini series are contained in Table 7.

Table 7: Diastereomeric ratios of compounds **29** – **32**.

Compound	29	30	31	32
d.r.	2.29 : 1.00	2.47 : 1.00	2.26 : 1.00	2.08 : 1.00

Compounds **29** and **31** showed diastereomeric ratios which were virtually equal. Since adamantyl isocyanide is achiral, it might have no effect on the diastereoselectivity of the reaction, however it is a very bulky isocyanide. The D/D and L/L combination of the other starting material for **29** and **31** would have an equal effect on the d.r., as although they are opposite enantiomers, they would have

the same impact on the reaction mechanism. We expected that this would also be true for **30** and **32**, since they are also enantiomers. However **30** displays a significantly greater d.r., and **32** the lowest value of the series.

Prior to characterising the products spectroscopically, we separated the compounds using preparative HPLC techniques. For this semi-preparative instrument, a large C-18 reverse phase column was used. The method utilised to achieve separation on the analytical HPLC was transferable to the semi-preparative system, which was advantageous. Despite this, the separation procedure involved multiple runs of the sample until the desired amount of pure diastereomer was obtained. The flow rate required was 20 ml/ minute, which is 20 times faster than that of the conventional HPLC, implying that large quantities of HPLC-grade solvent had to be used which is uneconomical. In addition, only limited quantities of compound could be separated at each run as highly concentrated samples resulted in no baseline separation. Nonetheless, we succeeded in separating the diastereomeric mixtures **29** – **32**, applying the gradient elution system mentioned previously. Eight pure diastereomers were isolated (Figure 29). To establish the stereochemistry of the major and minor diastereomers, we attempted to acquire crystals of the compounds. Unfortunately, this proved unsuccessful for any of the separated compounds. The findings by Zimuwandeyi concerning similar Passerini compounds (*N*-Boc-L-phenylalaninal, adamantyl isocyanide and propionic acid) to that of this project, show from crystal structures that the new stereogenic centre generated from the reaction for the major diastereomer was an *R*-centre when L-phenylalaninal was used.⁸⁰ Hence for their library of Passerini compounds, they assigned the major diastereomer as *R* and the minor one as *S* configuration at the newly formed stereocentre. Since the diastereoselectivity of the Passerini reaction was found to be largely dictated by the chiral aldehyde, applying the unnatural D-phenylalaninal in the reaction would result in the opposite product being formed, with the major diastereomer having an *S* configuration at the newly formed stereogenic centre and *R* for the minor one. Thus for this project, in the absence of other data, we will infer the stereochemistry based on the work of Zimuwandeyi for the major and minor diastereomers for this series.⁸⁰

Infrared spectroscopy predicts significant NH and C=O signals for nearly all the compounds. The NH peak for **31A** and **31B** is not clear, but emerges at 3200 – 3400 cm⁻¹ for the remainder of the products. The number of strong C=O stretches vary for each of the pure diastereomers. **30A**, **30B** and **32A** display four well-defined carbonyl stretches in the 1750 – 1640 cm⁻¹ range, which corresponds to the ester, amide and two Boc-entity C=O functionalities. The other Passerini adducts exhibit only three carbonyl group signals, whilst **29B** shows only two, which means that certain signals are overlapping.

The ^1H NMR spectra validated the synthesis and effective separation of all the Passerini compounds. Multiplet peaks for the aromatic protons for each of the compounds appeared at $\sim 7.60 - 6.95$ ppm. Unfortunately, the majority of the products integrated for slightly more than the 10 expected phenyl protons, possibly due to contaminants. Proton peaks for the adamantyl group appeared as expected. H-15, however, appeared as a singlet at ~ 1.66 ppm for selected compounds, but emerged as a multiplet for **29B**, **31A** and **31B**. From Table 8, it is evident that the NH and H-2 peaks appear differently for some of the separated products. Compounds **29A**, **31A** and **32B** reflect individual peaks for each of the NH and H-2 protons, whereas these signals overlap for the other compounds. Additionally, the minor diastereomers (**B**) of **29**, **31** and **32** show two singlet peaks for one of the NH protons found more upfield, as opposed to a single singlet for the rest of the products. A noteworthy observation was that C-2 appeared slightly more downfield in the ^{13}C NMR spectra for the minor diastereomers of this series except for compound **30**. This was also experienced by Zimuwandeyi for the newly formed stereogenic centre of separated Passerini diastereomers. This reinforces the notion that the configuration of the major and minor diastereomers established by Zimuwandeyi can also be attributed to our compounds.⁸⁰ As **29** (**A** and **B**) and **31** (**A** and **B**), and **30** (**A** and **B**) and **32** (**A** and **B**) are pairs of enantiomers, we expect their characterisation data to be identical. Considering the C-2 values (Table 8), the values for each of the enantiomers are virtually equal. However, the values of the enantiomers **29B** and **31B** and **30B** and **32B** differ somewhat. We noticed that there was a slight change in the shift values of C-2 (by roughly 0.2 ppm) from the ^{13}C NMR data obtained from the mixture of diastereomers prior to separation by preparative HPLC and the separated diastereomers. This indicates that it is possible for the shift values to differ slightly under variable conditions which could be the result of the disparities observed with the C-2 values of the aforementioned compounds.

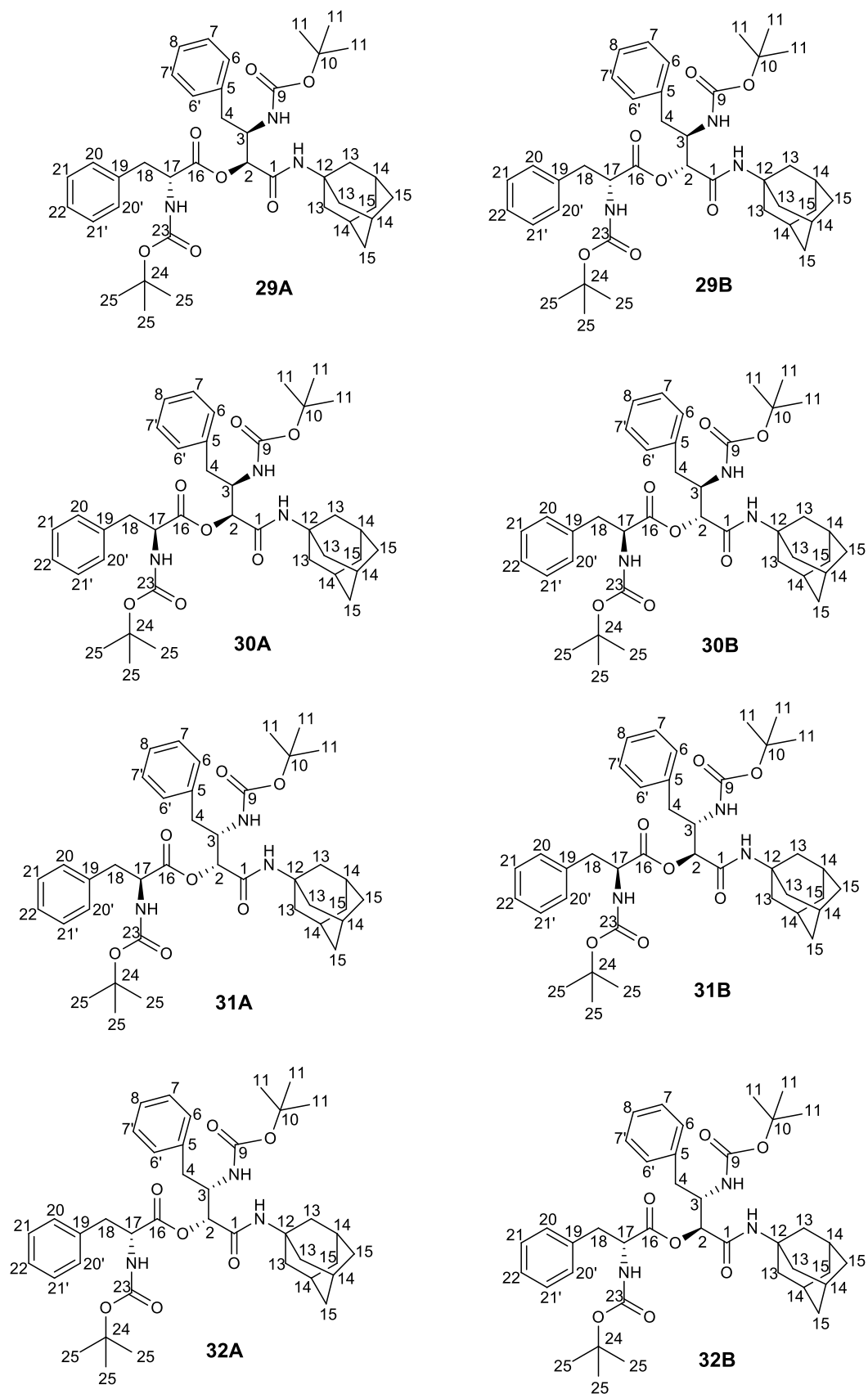


Figure 29: Adamantyl isocyanide series of pure Passerini diastereomers.

Table 8: NMR shifts of Passerini compounds from the adamantyl isocyanide series.

	3 x NH and H-2 / C-2		H-3 and H-17	H-4 and H-18 / C-4 and C-18	
	¹ H NMR	¹³ C NMR	¹ H NMR	¹ H NMR	¹³ C NMR
29A	δ 6.30 (1H, s, NH), 5.22 (1H, d, $J = 9.5$ Hz, NH), 5.14 – 4.97 (1H, m, H-2), 4.96 – 4.86 (1H, m, NH)	δ 74.32	δ 4.35 – 4.25 and 4.24 – 4.11 (2H, 2xm)	δ 3.22 – 2.90 and 2.65 – 2.44 (4H, 2xm)	δ 37.79 and 37.33
29B	δ 6.32 and 6.01 (1H, 2xs, NH), 5.37 – 4.78 (3H, m, 2xNH and H-2)	δ 75.74	δ 4.67 – 4.05 (2H, m)	δ 3.37 – 2.37 (4H, m)	δ 37.77 and 37.30
30A	δ 6.11 (1H, s, NH), 5.21 – 4.98 (2H, m, NH and H-2), 4.90 – 4.74 (1H, m, NH)	δ 74.76	δ 4.61 – 4.42 and 4.40 – 4.20 (2H, 2xm)	δ 3.27 – 2.94 and 2.86 – 2.51 (4H, 2xm)	δ 38.07 and 37.40
30B	δ 6.30 (1H, s, NH), 5.40 – 5.22 and 5.17 – 4.96 (2H, 2xm, NH and H-2), 4.50 – 4.07	δ 74.73	δ 4.50 – 4.07 (2H, 3xm)	δ 3.21 – 2.96 and 2.82 – 2.60 (4H, 2xm)	δ 38.03 and 37.33

	(1H, 3xm, NH)				
31A	δ 6.30 (1H, s, NH), 5.23 (1H, d, $J = 9.0$ Hz, NH), 5.16 – 5.01 (1H, m, $H-2$), 5.01 – 4.85 (1H, m, NH)	δ 74.26	δ 4.38 – 4.23 and 4.29 – 4.09 (2H, 2xm)	δ 3.14 – 2.92 and 2.66 – 2.47 (4H, 2xm)	δ 37.74 and 37.28
31B	δ 6.29 and 6.00 (1H, 2xs, NH), 5.31 – 5.00 (2H, m, NH and $H-2$), 4.99 – 4.76 (1H, m, NH),	δ 74.27	δ 4.61 – 4.10 (2H, m)	δ 3.24 – 2.72 and 2.63 – 2.49 (4H, 2xm)	δ 37.75 and 37.29
32A	δ 6.10 (1H, s, NH), 5.21 – 4.96 (2H, m, NH and $H-2$), 4.89 – 4.74 (1H, m, NH),	δ 74.73	δ 4.60 – 4.43 and 4.39 – 4.21 (2H, 2xm)	δ 3.27 – 2.94 and 2.87 – 2.57 (4H, 2xm)	δ 38.02 and 37.35
32B	δ 6.34 and 6.10 (1H, 2xs, NH), 5.40 – 5.23 (1H, m, $H-2$), 5.16 – 5.01 (1H, m, NH) 4.57 – 4.06 (1H, 3xm, NH)	δ 75.22	δ 4.57 – 4.06 (2H, 3xm)	δ 3.22 – 2.94 and 2.83 – 2.58 (4H, 2xm)	δ 38.01 and 37.76

The melting points for this series show an interesting pattern. From Table 9, each of the pairs of diastereomers have a melting point that is markedly similar. However, **32A** and **32B** are the only exception as their melting points differ substantially. Compounds **29A** and **29B** and **31A** and **31B** have similar melting points as expected as they are pairs of enantiomers. Compounds **30** (**A** and **B**) and **32** (**A** and **B**) show considerably greater values however, **30B** and **32B** have significantly different melting points.

Table 9: Melting points of compounds **29 – 32**.

Compound	29A	29B	30A	30B	31A	31B	32A	32B
m.p. (°C)	92 - 94	86 - 88	181 - 183	185 - 187	79 - 80	80 - 82	192 - 195	102 - 104

2.3.6 Diastereoselectivity of the D-phenylalanine isocyanide series

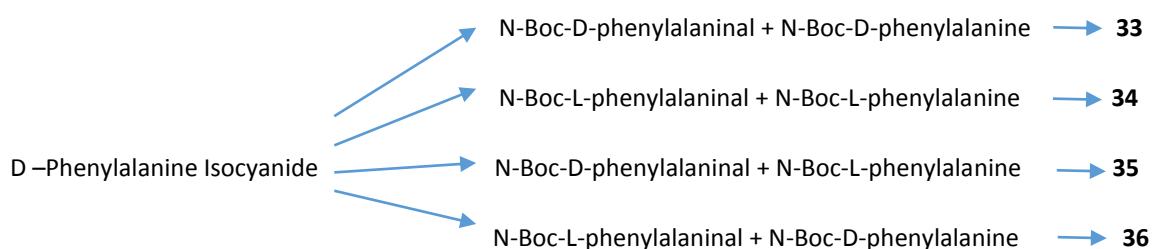


Figure 30: Synthetic plan for Passerini diastereomers using D-phenylalanine isocyanide.

With success in the preparation and separation of the adamantyl isocyanide sequence, we progressed to applying the chiral D-phenylalanine isocyanide in the reaction and monitored the influence on the d.r. The reactions were conducted in the same manner stated previously using 0.46 mmol of each of the starting materials. Again, four reactions were carried out as shown in Figure 30. After purification, compounds **33 – 36** (Figure 31) were obtained as a mixture of two diastereomers in yields ranging between 42 – 80%. Developing a separation method on the C-18 column for this collection of compounds was less straightforward than that described in 2.3.5. Using a gradient elution of 60:40 to 65:35 acetonitrile/ water ultimately allowed determination of the d.r. for these compounds with lengthy elution times just below 60 minutes.

Table 10: Diastereomeric ratios of compounds **33** – **36**.

Compound	33	34	35	36
d.r.	1.97 : 1.00	2.46 : 1.00	1.80 : 1.00	1.94 : 1.00

Table 10 indicates a d.r. of approximately 2:1 for **33** and **36**, whilst **34** and **35** are outliers. With a result of 2.46 : 1.00, **34** displays the highest d.r. indicating preference of one diastereomer over the other. Hence this would be “matched” because it leads to greater diastereoselectivity. On the other hand, compound **35** is a “mismatch” as it led to a decrease in diastereoselectivity. This denotes that the use of chiral isocyanides does indeed play a role in the diastereoselectivity of the reaction, in addition to the combination of chiral acids and aldehydes involved.

We moved on to separate the diastereomers using semi-preparative HPLC with the same method applied on the C-18 column. Since these runs persisted for nearly an hour, liberal amounts of HPLC solvent were expended and only minute quantities of the compounds were collected for further characterisation. Unfortunately, we were unable to obtain crystals for this series, and so the stereochemistry of this set of compounds was also extrapolated as per Zimuwandeyi’s results, with the major diastereomer being *R* and the minor one being *S* at the new stereogenic centre for compounds prepared with L-phenylalaninal.⁸⁰ On the other hand, the opposite product was obtained when D-phenylalaninal was employed.

Upon analysis of the ¹H NMR spectra, synthesis of all eight compounds was confirmed. For the major diastereomer of **33** (**A**), the aromatic rings appeared as a multiplet at 7.38 – 7.09 ppm integrating for 17 protons (greater than the expected 15H due to probable contamination). The H-12 signal overlapped with the peak consistent with H-2 as a multiplet at 5.35 – 5.11 ppm. The three NH signals were reflected further upfield as three separate multiplets. The very similar, H-22 and H-3 occurred as two multiplets between 4.52 - and 4.10 ppm with an integration of 2H. However, H-12 which is also similar to H-22 and H-3 appeared more downfield overlapped with H-2 as noted from 2D NMR data as well as from the NMR data of Zimuwandeyi for compounds containing similar starting material to that of our products.⁸⁰ The Boc-groups appeared as a multiplet at the predicted position, with H-20 arising as a singlet at 3.62 ppm.

All seven ¹H NMR spectra of the other pure diastereomers resembled that of **33A**, apart from the two NH, H-12 and H-2 signals which did not appear as separate peaks for selected products. Additionally H-20 appeared as a multiplet for **33B**, **34A**, **35B** and **36** (**A** and **B**) instead of the predicted singlet.

The ^{13}C NMR spectra further validated the formation of the above compounds. Since only single peaks for each carbon environment were observed (in contrast to doubling of peaks for a mixture of compounds), the separation of the diastereomers also proved successful. Five carbonyl peaks were seen due to the ester, two amide and two Boc-carbamate functionalities for each of the adducts. C-22, C-12 and C-3 appear between 56- and 50.00 ppm. Similarly C-23, C-13 and C-4 all appear in a close range of 38 – 37.00 ppm. As with the adamantyl isocyanide series, C-2 is observed more downfield for the minor diastereomers (**33B** – **36B**) (Table 11). Compound **36B** was the only exception in this case.

Table 11: ^{13}C NMR shifts for C-2 of compounds **33** – **36**.

Compound	33A	33B	34A	34B	35A	35B	36A	36B
δ C-2	73.97	74.19	74.04	74.19	74.46	75.03	75.09	75.05

The NH and C=O stretches were key from the infrared data. Three carbonyl peaks were noted for each of the separated diastereomers as presented in Table 12, conforming to the carbamate, amide and ester functional groups.

Table 12: Major IR peaks of compounds **33** – **36**.

Compound	33A	33B	34A	34B	35A	35B	36A	36B
NH stretch (cm^{-1})	3358	3352	3349	3358	3309	3322	3313	3312
C=O stretches (cm^{-1})	1756, 1691, 1660	1756, 1689, 1659	1755, 1689, 1656	1755, 1691, 1656	1746, 1693, 1658	1756, 1689, 1660	1753, 1695, 1656	1753, 1692, 1656

Mass spectroscopy also verified preparation of **33** – **36**.

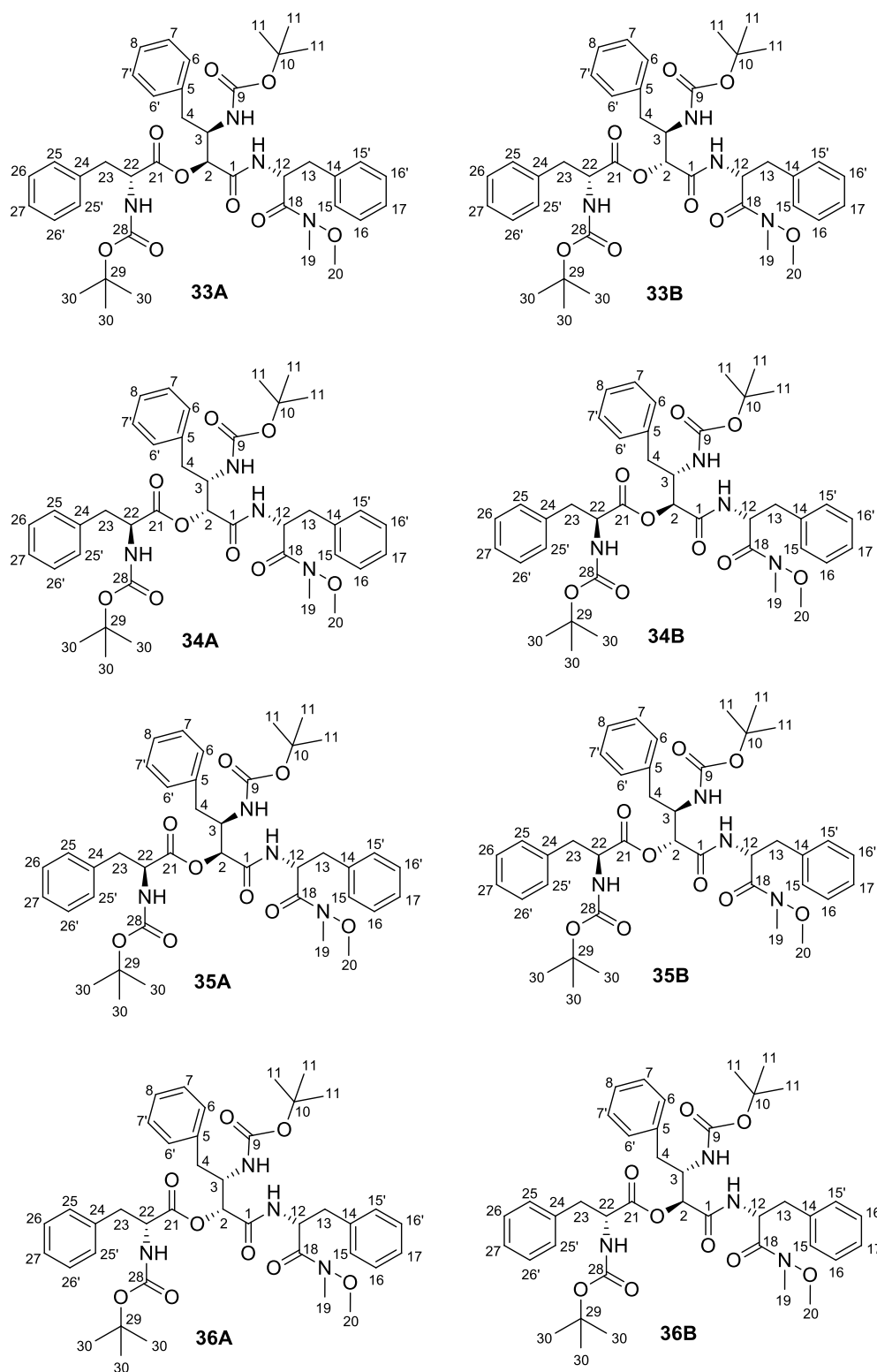


Figure 31: D-phenylalanine isocyanide series of pure Passerini diastereomers.

2.3.7 Diastereoselectivity of the L-phenylalanine isocyanide series

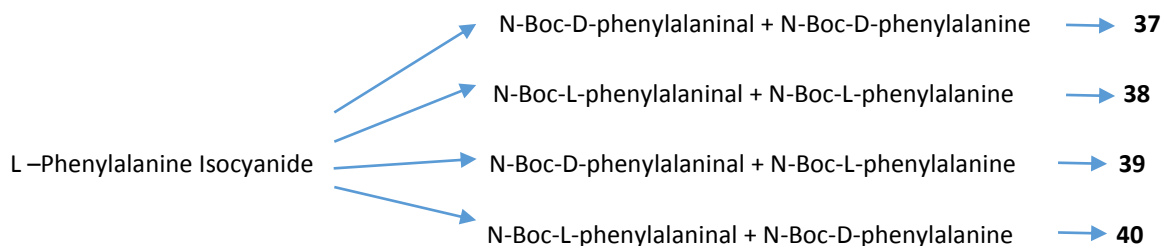


Figure 32: Synthetic plan for Passerini diastereomers using D-phenylalanine isocyanide.

The final collection of diastereomers were prepared with the chiral L-phenylalanine isocyanide (Figure 32). Compounds **37** – **40** (Figure 33) were achieved in an excellent average yield of 88%. An identical method for both the C-18 and semi-preparative separation was employed for this set as for the D-phenylalanine isocyanide group. The d.r. values for these compounds are approximately 2:1 (Table 13). Nonetheless no significant pattern is displayed with the diastereoselectivity, even for the compounds prepared with D/D, L/L and D/L, L/D acids and aldehydes. Hence for this series, no conclusion can be made on whether the various combination of chiral starting material in the Passerini reaction largely impacts the d.r. Once more, the configuration of the major and minor diastereomers were assigned according to that of Zimuwandeyi as acquisition of crystals for these separated compounds were problematic.⁸⁰

Table 13: Diastereomeric ratios of compounds **37** – **40**.

Compound	37	38	39	40
d.r.	1.90 : 1.00	1.78 : 1.00	1.85 : 1.00	2.03 : 1.00

The ¹H NMR data for this separated series is virtually identical to the aforementioned series. The phenyl ring protons for all 8 diastereomers appear at ~7.50 – 6.95 ppm as a multiplet with an integration of greater than 15H. All three Passerini series reflect a larger number of aromatic protons than predicted, the most likely reason being contamination. For some compounds the NH, H-12 and H-2 signals appeared as individual peaks, whereas in others they overlapped. Just as for the series in **2.3.6**, H-22 and H-3 appear alongside each other but H-12, although it is remarkably similar appeared slightly more downfield. The signals for H-4, H-13, H-19 and H-23 appear together as a multiplet for

most of the compounds at ~3.30 – 2.40 ppm. The Boc-protons H-11 and H-30 are observed as a multiplet ~1.60 – 1.15 ppm and integrate for 18 expected protons. ¹³C NMR spectroscopy displayed each of the peaks for all the compounds as anticipated. Table 14 shows C-2 for both the major and minor diastereomers. As predicted from the work of Zimuwandeyi and the results obtained from the previous series, the major diastereomers (**A**) reflect further upfield as compared to the minor counterpart - indicating that the stereochemistry of both diastereomers are correctly assumed.⁸⁰ Compound **39A** however does not fit the predicted result.

Table 14: ¹³C NMR shifts for C-2 of compounds **37 – 40**.

Compound	37A	37B	38A	38B	39A	39B	40A	40B
δ C-2	74.13	74.19	73.97	75.05	75.09	75.03	74.46	75.05

Infrared spectroscopy for each of the separated diastereomers of this series display an NH and three strong C=O stretches, analogous to the D-phenylalanine isocyanide group. The melting points of **37** and **38** (**A** and **B**) prepared using a mix of D/D and L/L aldehyde and acid, respectively, were considerably higher (98 – 139 °C) to that of compounds **39** and **40** (**A** and **B**) at 65 – 74 °C, which were synthesised with a mixture of D/L and L/D aldehydes and acids, respectively.

Table 15: ¹³C NMR shifts for C-2 for the sets of enantiomers of compounds **33 – 40**.

Compound	δ C-2	Compound	δ C-2
33A	73.97	38A	73.97
33B	74.19	38B	75.05
34A	74.04	37A	74.13
34B	74.19	37B	74.19
35A	74.46	40A	74.46
35B	75.03	40B	75.05
36A	75.09	39A	75.09
36B	75.05	39B	75.03

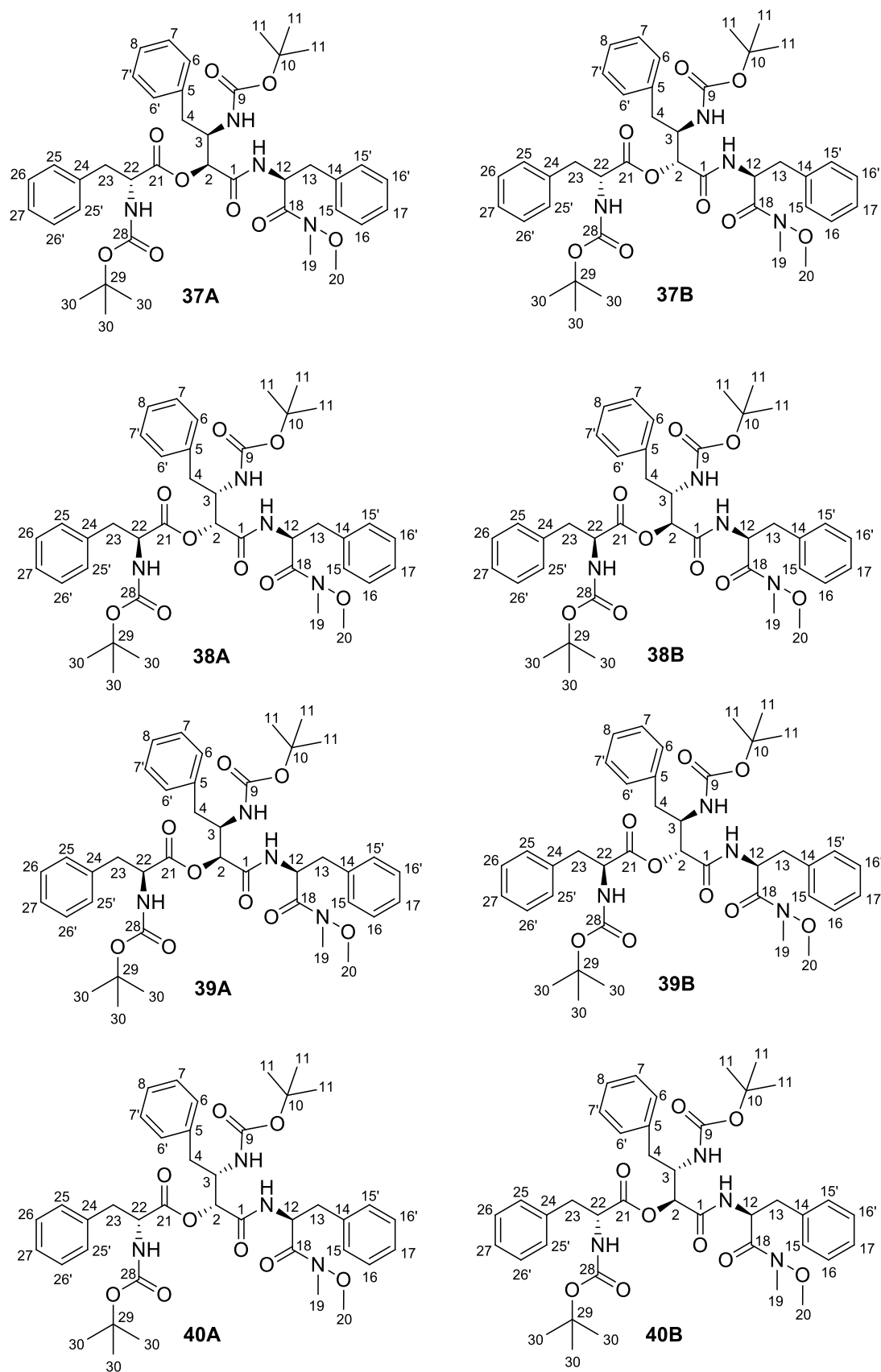


Figure 33: L-phenylalanine isocyanide series of pure Passerini diastereomers.

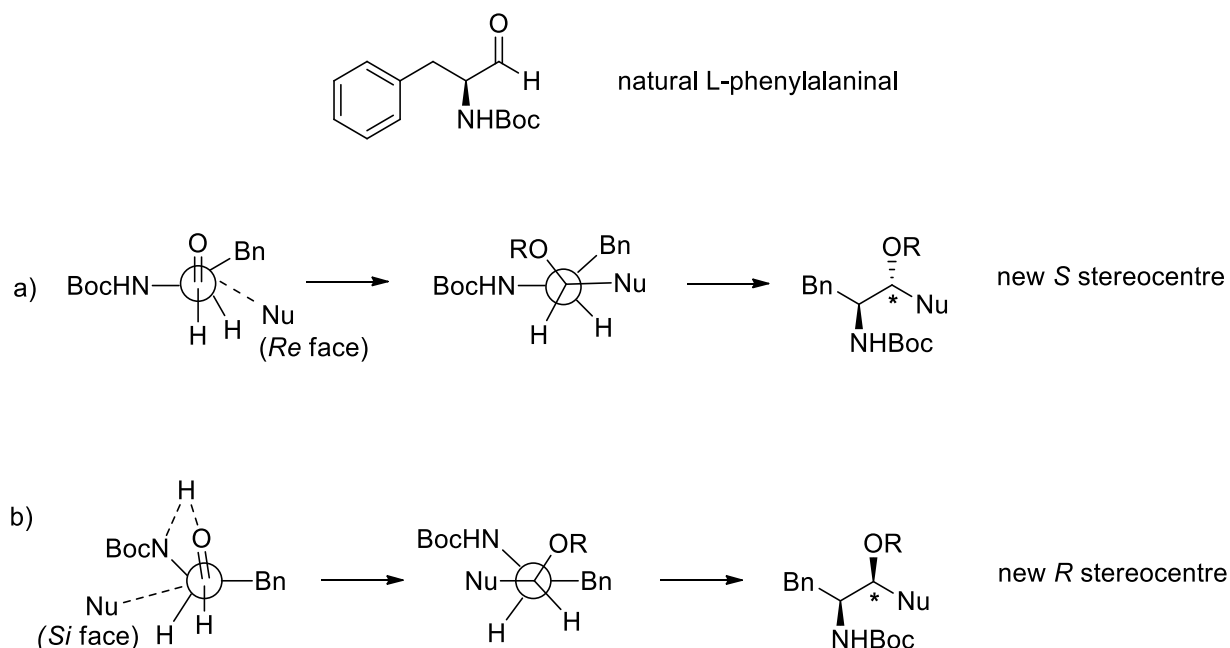
From the D-phenylalanine isocyanide and L-phenylalanine isocyanide series of the Passerini diastereomers, it is evident that sets of enantiomers exist between the two groups. For instance, compound **33** (**A** and **B**) is an enantiomer of **38** (**A** and **B**) as they were synthesised using a combination of D/D/D and L/L/L isocyanide/aldehyde/acid reagents, respectively. Likewise, **34** and **37**, **35** and **40** and **36** and **39** are also sets of enantiomers. Hence we expect the characterisation data of these pairs of compounds to be identical. From ^{13}C NMR spectroscopy, the C-2 values of the major (**A**) and the minor (**B**) diastereomers of these four sets of enantiomers are virtually equal as predicted (Table 15). The only exception is **33B** and **38B**, for which the C-2 values differ substantially (74.19 and 75.05 ppm). As seen with the adamantyl isocyanide series, the shift values differed slightly when the samples were run as a diastereomeric mixture in comparison to the separated diastereomers, making it possible for the C-2 values to be slightly different. As noted previously with our results and with that noticed by Zimuwandeyi, the C-2 values of the minor diastereomers appeared more downfield in comparison to the major diastereomer.⁸⁰ What is interesting is that the outlier from the D-phenylalanine isocyanide series was compound **36**, and that from the L-phenylalanine isocyanide collection was **39**, and these compounds are found to be a set of enantiomers. Hence, this indicates a definite relationship between compounds prepared with certain combinations of chiral starting materials in the Passerini reaction.

2.3.8 Proposed model for the configuration and diastereoselectivity of the Passerini reaction

From the results obtained it is evident that the d.r. values of the diastereomeric compounds in each of the three series are close to 2:1. However, the D and L-phenylalanine isocyanide series reflect values slightly lower than the average, whilst the adamantyl isocyanide series display values greater than 2:1 for all four pairs of diastereomers (**29** – **32**). Zimuwandeyi found that the chiral aldehyde (phenylalaninal) largely dictates the diastereoselectivity of the Passerini reaction, but from our results it is apparent that the type of isocyanide also plays a role in the d.r. values of the compounds.⁸⁰

To rationalise the configuration of the newly generated stereocentre in the Passerini reaction, Zimuwandeyi proposed that the reaction followed a chelation controlled pathway instead of the Felkin-Anh model.⁸⁰ Section 1.2.2 outlines the Passerini reaction mechanism in aprotic solvents which involves H-bonding between the acid and aldehyde, followed by addition of the nucleophilic isocyanide. Since the aldehyde is chiral in this case, the nucleophile can approach it by either the *Re* or *Si* face. According to the Felkin-Anh mechanism, the isocyanide would attack the *Re* face to minimise steric hindrance due to the large mono-substituted amino aldehyde, giving rise to an *S* configuration at the newly formed stereocentre of the major diastereomer when the natural L-phenylalaninal is used (Scheme 37). The opposite configuration would be achieved when D-phenylalaninal is employed. However, Zimuwandeyi realised from crystal structures that the

compounds exhibited the stereochemistry opposite to that suggested by the Felkin-Anh model.⁸⁰ This led to the conception that intramolecular H-bonding that occurs between the carbonyl oxygen and the NH of the aldehyde, allowed the reaction to adhere to the chelation model which is further described by Hili.⁹⁴ This arranges the aldehyde in a fixed position in which the large phenyl group lies perpendicular to the carbonyl moiety, making the *Si* face the most favourable for the nucleophile to attack in cases when L-phenylalaninal is used (Scheme 37). The result would be an *R* configuration for the major diastereomer at the new stereocentre, which substantiates the data obtained by Zimuwandeyi.⁸⁰ Once more, the opposite configuration is achieved when D-phenylalaninal is applied, resulting in an *S*-centre for the major product. The stereochemistry of the diastereomers obtained in our project was based on the findings by Zimuwandeyi, as our compounds were non-crystalline hence obtaining crystal structures was not possible.⁸⁰



Scheme 37: Mechanism of the Passerini reaction with L-phenylalaninal, indicating formation of the major diastereomer: a) Felkin-Anh model b) chelation controlled model.

Generally, the size of the nucleophile significantly influences the stereoselectivity of the reaction, with larger nucleophiles resulting in superior diastereoselectivity. Since H-bonding occurs between two moieties of the aldehyde, fixing the arrangement of the transition state, the nucleophile (isocyanide) attacks the face which results in the least steric hindrance. Adamantyl isocyanide is especially bulky as

compared to the D and L-phenylalanine isocyanides, hence causing a slightly greater discrimination with the diastereoselectivity. As a result, the d.r. values for the branched adamantyl isocyanide series exceeded the average 2:1 ratio. The results obtained in this project are consistent with those found by Zimuwandeyi, where larger isocyanides somewhat enhanced the diastereoselectivity of the Passerini reaction as opposed to smaller isocyanides.⁸⁰

Chapter 3: Conclusions and Future work

3.1 Conclusions

We endeavoured to synthesise peptidomimetic compounds using MCRs. The racemic mixtures obtained from these reactions were suitable for separation by means of EKR to generate pure products with prospective bioactivity.

A library of enantiomeric adducts were prepared via the isocyanide-based U-4CR MCR employing a variety of carboxylic acids. As methanol and ammonia were utilised in the reaction, extremely low yields of all the compounds were obtained as predicted. Altering the solvent to the less nucleophilic 2,2,2-trifluoroethanol increased the yields by nearly threefold. Proteases and penicillin G acylase were then applied to the racemates in order to resolve them by hydrolysis of the amide bonds. Regrettably, the enzymes failed to hydrolyse the Ugi-compounds and showed no chemical transformation under various reaction conditions. Also these compounds exhibited poor solubility in organic solvents which could also contribute to the failure of EKR.

Using a range of aldehydes, enantiomeric compounds were produced by the Passerini MCR reaction. This time we opted to resolve the mixtures using lipases 6Y and 6Z. These compounds displayed greater solubility than the previous set in most organic solvents. Enzymatic reactions were performed on these compounds and both lipases effectively hydrolysed the products in a short period of time. With the success experienced using the biocatalysts, we aimed to determine the enantioselectivity of the EKR reactions using chiral HPLC. The Passerini benzaldehyde-derivative portrayed moderate conversion and selectivity by both lipases as ascertained by the AD-H chiral column. Failure to obtain the necessary information to investigate the selectivity of the other Passerini enantiomers by chiral HPLC, led us to conduct the Mosher ester analysis. From ^{19}F NMR, we established that EKR of the racemic hydrocinnamaldehyde-derivative exhibited good enantioselectivity and that they could effectively be separated through biocatalysts. On the other hand, no data regarding selectivity of the Passerini compound formed by *trans*-cinnamaldehyde could be deduced either by chiral columns or by reaction with Mosher's acid. In essence, we have successfully demonstrated that EKR is an effective technique for the separation of racemic mixtures. Greater enantioselectivity can be achieved in future by modification of reaction conditions, enzyme (via enzyme engineering) and the substrate involved.

In addition to EKR of peptide mimics, we also set out to investigate the diastereoselectivity of the Passerini reaction. Previous studies have shown that the involvement of starting material in this MCR which contains a fixed stereogenic centre, plays a significant role in the d.r of the reaction. We

prepared three series of Passerini diastereomers using three different isocyanides, namely adamantyl, D-phenylalanine- and L-phenylalanine isocyanides, in combination with chiral Boc-D- or L-phenylalanine and Boc-D- or L-phenylalaninal. Four diastereomeric mixtures were obtained in each group and the d.r. was close to 2:1 for most products.

Prior to characterisation of these mixtures, separation by semi-preparative HPLC was undertaken to generate eight pure diastereomers in each Passerini series. The stereochemistry of the diastereomers could not be established by X-ray crystallography as the compounds were not crystalline hence, by analogy to previous work by Zimuwandeyi we concluded the configuration of the newly formed stereogenic centre of the major diastereomer to be *R* and *S* for that of the minor diastereomer when L-phenylalaninal was employed as the aldehyde component. Since the diastereoselectivity of the Passerini reaction is significantly affected by the aldehyde, the opposite product was obtained when D-phenylalaninal was used, with the major diastereomer having an *S* configuration for the newly formed stereocentre and *R* for the minor one. ^{13}C NMR spectroscopy of the separated compounds showed distinct patterns for particular environments which reinforced the proposed configurations. The peaks from the ^{13}C NMR spectra of only two compounds from the D- and L-phenylalanine series did not conform to this.

The d.r. values obtained from this study indicated that the use of chiral starting material does in fact impact the diastereoselectivity of the Passerini reaction. The use of a D/D, L/L or D/L, L/D combination of reagents seem to influence the d.r. in most cases, probably due to the effect of steric hindrance. The Felkin-Anh chelation controlled mechanism rationalises the stereochemistry of the major and minor diastereomers. The size of the isocyanide evidently affects the diastereoselectivity of the Passerini reaction, with the bulky adamantyl isocyanide series displaying the greatest d.r. values in comparison to the other two series. However further studies must be carried out to resolve the inconsistencies as not all the data suggest a clear significant trend with the various combinations of starting materials.

3.2 Future work

Given that no positive result was gained by EKR of the Ugi-enantiomers, it would be interesting to investigate the diastereoselectivity of this reaction by the introduction of chiral starting material. The Mosher ester analysis of the *trans*-cinnamaldehyde Passerini derivative **13** would have to be reconsidered in order to ascertain the enantioselectivity of the EKR of this compound. If not, a different method could be tested to gain insight into the selectivity of this reaction. The absolute configuration of the Passerini enantiomers (**12** – **14**) could also be discovered in order to distinguish which enantiomer is preferably being hydrolysed by Lipases 6Y and 6Z. Since no definite conclusions could be drawn from the trend using certain combinations of chiral starting materials on the diastereoselectivity of the Passerini reaction, different chiral isocyanides (*eg.* glucose isocyanide) can be synthesised and the effect on the d.r. can be determined. In this way we can deduce how different chiral starting materials participate in the reaction and hence predict which combinations would lead to preference of one diastereomer over the other. Additionally the pure Passerini diastereomers isolated can be subjected to biological testing to establish if they display any promise of bioactivity.

Chapter 4: Experimental Procedures

4.1 General Experimental Procedures

4.1.1 Purification of Reagents and Solvents

All reagents and HPLC solvents were purchased from commercial suppliers and used without further purification. Solvents used for preparative chromatography were distilled before use. For reaction purposes, solvents were stored over activated molecular sieves (4 Å) and then distilled over the appropriate drying agent under a nitrogen atmosphere. THF was distilled from sodium wire with benzophenone as an indicator. CH₂Cl₂ was distilled from calcium hydride.

4.1.2 Chromatography

Thin layer chromatography was performed on aluminium-backed Macherey-Nagel UV₂₅₄ plates pre-coated with silica gel 60, and was visualised under UV light or by using iodine, ninhydrin or vanillin staining solutions. Column chromatography was carried out using Fluka silica gel 60 (particle size 0.063 – 0.200 mm). Analytical HPLC was performed on a Dionex Ultimate 3000 pump using a Luna 5u C18 (2) reverse phase column with gradient elution of MeCN/H₂O for diastereomers and a Chiralpak AD-H or Lux 5 µm Amylose-2 chiral column with isocratic elution of IPA/Hexane for enantiomers (flow rate of 1 ml/min at 217 nm). Preparative HPLC was carried out using an Agilent 3000 instrument with an Agilent prep C18 reverse phase column (21.2 x 20.0 mm) with gradient elution of MeCN/H₂O (flow rate of 20 ml/min at 217 nm). All calculations were based on peak area.

4.1.3 Spectroscopic and Physical Data

Optical rotations were measured on a Jasco P-2000 Polarimeter at 20 °C at the sodium D line (589 nm).

Melting points were determined using a Stuart SMP10 apparatus and are uncorrected.

Infrared spectra were obtained using a Bruker Tensor 27 Fourier Transform spectrometer fitted with a diamond ATR device.

¹H NMR and ¹³C NMR spectra were recorded on a Bruker AVANCE 300 spectrometer at 300 MHz and 75 MHz, respectively unless stated otherwise. Chemical shifts are reported in parts per million (ppm) relative to the internal standard, tetramethylsilane for ¹H and CDCl₃ (77.00 ppm) for ¹³C.

HRMS data was obtained using a Waters Synapt G2 spectrometer.

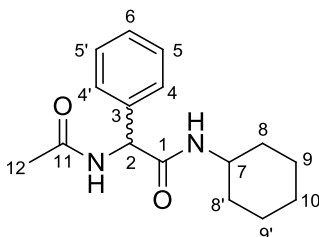
4.2 Enzymatic Kinetic Resolution of U-4CR Enantiomers

4.2.1 Synthesis of U-4CR Enantiomers

4.2.1.1 Synthesis of 2-acetamido-*N*-cyclohexyl-2-phenylacetamide **7**

Benzaldehyde (305 μ l, 3.00 mmol) was added to a solution of ammonia (224 μ l of a 25% solution, 3.00 mmol) in MeOH (3 ml) and the mixture was stirred for 15 min. Acetic acid (172 μ l, 3.00 mmol) was then added and the reaction stirred for a further 5 min after which cyclohexyl isocyanide (373 μ l, 3.00 mmol) was added. The mixture was left to stir overnight at room temperature. Excess solvent was removed *in vacuo* and the residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1:0 to 9:1) to afford **7** (0.10 g, 12%) as a white solid.

The synthesis of compound **7** was repeated as described above except using 2,2,2-trifluoro-ethanol as the solvent (0.33 g, 40%).

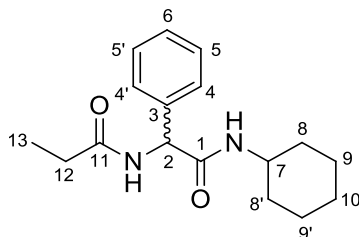


R_f = 0.35 (5% MeOH/ CH_2Cl_2); *m.p.* 243 – 245 $^{\circ}\text{C}$; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3280 (NH), 2935, 2855 (C-H), 1633 (C=O), 1540 (C=C), 1058 (C-N); ^1H NMR (300 MHz, CDCl_3) δ 7.47 – 7.15 (5H, m, Ar-H), 6.97 (1H, d, J = 6.8 Hz, NH), 5.86 (1H, d, J = 8.0 Hz, NH), 5.53 – 5.37 (1H, m, H-2), 3.84 – 3.50 (1H, m, H-7), 2.01 (3H, s, H-12), 1.97 – 1.45 (5H, m, $\text{CH}_{2\text{eq}}$), 1.44 – 0.88 (5H, m, $\text{CH}_{2\text{ax}}$); ^{13}C NMR (75 MHz, CDCl_3) δ 169.62 and 169.12 (C-11 and C-1), 138.45 (C-3), 128.89 (C-5, C-5'), 128.17 (C-6), 127.17 (C-4, C-4'), 56.90 (C-2), 48.71 (C-7), 32.73 and 32.55 (C-8, C-8'), 25.42 (C-10), 24.69 and 24.57 (C-9, C-9'), 23.20 (C-12); HRMS *m/z*: calculated for $\text{C}_{16}\text{H}_{23}\text{N}_2\text{O}_2$: 275.1760, found $[\text{M} + \text{H}]^+$: 275.1764.

4.2.1.2 Synthesis of *N*-(2-(cyclohexylamino)-2-oxo-1-phenylethyl)propionamide **8**

Benzaldehyde (305 μ l, 3.00 mmol) was added to a solution of ammonia (224 μ l of a 25% solution, 3.00 mmol) in MeOH (3 ml) and the mixture was stirred for 15 min. Propionic acid (225 μ l, 3.00 mmol) was then added and the reaction stirred for a further 5 min after which cyclohexyl isocyanide (373 μ l, 3.00

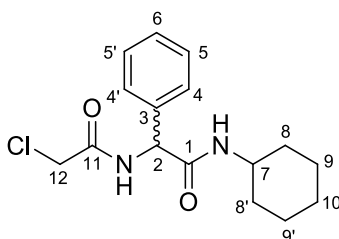
mmol) was added. The preparation was completed using the same procedure as described in **4.2.1.1** to afford **8** (0.03 g, 3%) as a white solid.



R_f = 0.43 (5% MeOH/CH₂Cl₂); **m.p.** 217 – 218 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3283 (NH), 2935, 2854 (C-H), 1634 (C=O), 1533 (C=C); **¹H NMR** (300 MHz, CDCl₃) δ 7.83 (0.2H, d, J = 8.0 Hz, NH), 7.47 – 7.32 and 7.29 – 7.21 (6H, 2xm, Ar-H and NH), 7.04 (0.8H, d, J = 8.0 Hz, NH), 5.78 (1H, d, J = 7.8 Hz, H-2), 3.76 – 3.58 (1H, m, H-7), 2.28 (2H, q, J = 7.6 Hz, H-12), 1.90 – 1.47 (5H, m, CH_{2eq}), 1.36 – 0.97 (8H, 2xm overlapped with t, J = 7.5 Hz, CH_{2ax} and H-13); **¹³C NMR** (75 MHz, CDCl₃) δ 173.21 and 169.15 (C-11 and C-1), 138.62 (C-3), 128.86 (C-5, C-5'), 128.11 (C-6), 127.14 (C-4, C-4'), 56.77 (C-2), 48.71 (C-7), 32.76 and 32.59 (C-8, C-8'), 29.72 and 29.52 (C-12), 25.43 (C-10), 24.73 and 24.62 (C-9, C-9'), 9.62 (C-13); **HRMS m/z**: calculated for C₁₇H₂₅N₂O₂: 289.1916, found [M + H]⁺: 289.1917.

4.2.1.3 Synthesis of 2-(2-chloroacetamido)-N-cyclohexyl-2-phenylacetamide 9

Benzaldehyde (305 μ l, 3.00 mmol) was added to a solution of ammonia (224 μ l of a 25% solution, 3.00 mmol) in MeOH (3 ml) and the mixture was stirred for 15 min. Chloroacetic acid (283 mg, 3.00 mmol) was then added and the reaction stirred for a further 5 min after which cyclohexyl isocyanide (373 μ l, 3.00 mmol) was added. The preparation was completed using the same procedure as described in **4.2.1.1** to afford **9** (0.14 g, 15%) as a white solid.



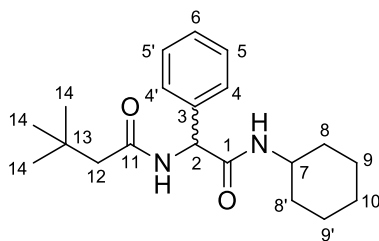
R_f = 0.56 (5% MeOH/CH₂Cl₂); **m.p.** 211 – 213 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3291 (NH), 2931, 2855 (C-H), 1633 (C=O), 1538 (C=C); **¹H NMR** (300 MHz, CDCl₃) δ 7.99 (1H, d, J = 6.4 Hz, NH), 7.48 – 7.26 (5H, m, Ar-H), 5.80 (1H, d, J = 7.8 Hz, NH), 5.44 (1H, d, J = 7.0 Hz, H-2), 4.02 (2H, s, H-12), 3.84 – 3.64 (1H, m, H-7), 1.96 – 1.47 (5H, m, CH_{2eq}), 1.40 – 0.86 (5H, m, CH_{2ax}); **¹³C NMR** (75 MHz, CDCl₃) δ 168.23 and 165.53 (C-11 and C-1), 137.71 (C-3), 129.10 (C-5, C-5'), 128.58 (C-6), 127.30 (C-4, C-4'), 57.26 (C-2), 48.92 (C-

7), 42.48 (C-12), 32.77 and 32.56 (C-8, C-8'), 25.38 (C-10), 24.69 and 24.56 (C-9, C-9'); **HRMS m/z**: calculated for $C_{16}H_{22}ClN_2O_2$: 309.1370, found $[M + H]^+$: 309.1358.

4.2.1.4 Synthesis of *N*-(2-(cyclohexylamino)-2-oxo-1-phenylethyl)-3,3-dimethylbutanamide **10**

Benzaldehyde (305 μ l, 3.00 mmol) was added to a solution of ammonia (224 μ l of a 25% solution, 3.00 mmol) in MeOH (3 ml) and the mixture was stirred for 15 min. *t*-Butylacetic acid (383 μ l, 3.00 mmol) was then added and the reaction stirred for a further 5 min after which cyclohexyl isocyanide (373 μ l, 3.00 mmol) was added. The preparation was completed using the same procedure as described in **4.2.1.1** to afford **10** (0.18 g, 18%) as a white solid.

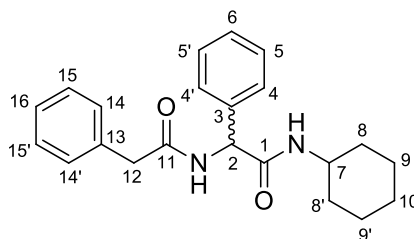
The synthesis of compound **10** was repeated as described above except using 2,2,2-trifluoro-ethanol as the solvent (0.69 g, 70%).



R_f = 0.44 (5% MeOH/ CH_2Cl_2); **m.p.** 231 – 233 °C; **IR** (ν_{max}/cm^{-1}): 3295 (NH), 2936, 2859 (C-H), 1628 (C=O), 1520 (C=C), 1059 (C-N); **1H NMR (300 MHz, $CDCl_3$)** δ 7.45 – 7.29 (5H, m, Ar-H), 6.81 (1H, d, J = 6.9 Hz, NH), 5.79 (1H, d, J = 8.0 Hz, NH), 5.46 (1H, d, J = 7.0 Hz, H-2), 3.86 – 3.62 (1H, m, H-7), 2.10 (2H, s, H-12), 2.04 – 1.82 and 1.79 – 1.48 (5H, 2xm, CH_{2eq}), 1.46 – 1.18 and 1.16 – 0.96 (5H, 2xm, CH_{2ax}), 0.99 (9H, s, H-14); **^{13}C NMR (75 MHz, $CDCl_3$)** δ 171.09 and 169.03 (C-11 and C-1), 138.65 (C-3), 128.74 (C-5, C-5'), 128.14 (C-6), 127.31 (C-4, C-4'), 56.93 (C-2), 50.38 (C-12), 48.72 (C-7), 32.78 and 32.61 (C-8, C-8'), 31.04 (C-13), 29.84 (C-14), 25.42 (C-10), 24.73 and 24.61 (C-9, C-9'); **HRMS m/z**: calculated for $C_{20}H_{31}N_2O_2$: 331.2386, found $[M + H]^+$: 331.2381.

4.2.1.5 Synthesis of *N*-cyclohexyl-2-phenyl-2-(2-phenylacetamido)acetamide **11**

Benzaldehyde (305 μ l, 3.00 mmol) was added to a solution of ammonia (224 μ l of a 25% solution, 3.00 mmol) in 2,2,2-trifluoro-ethanol (3 ml) and the mixture was stirred for 15 min. Phenylacetic acid (408 mg, 3.00 mmol) was then added and the reaction stirred for a further 5 min after which cyclohexyl isocyanide (373 μ l, 3.00 mmol) was added. The preparation was completed using the same procedure as described in **4.2.1.1** to afford **11** (0.15 g, 14%) as a white solid.



R_f = 0.59 (5% MeOH/CH₂Cl₂); **m.p.** 245 – 247 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3294 (NH), 2937, 2864 (C-H), 1632 (C=O), 1529 (C=C), 1053 (C-N); **¹H NMR (300 MHz, CDCl₃)** δ 7.46 – 7.24 (10H, m, Ar-H), 6.88 (1H, d, J = 6.8 Hz, NH), 5.48 (1H, d, J = 8.2 Hz, NH), 5.36 (1H, d, J = 6.8 Hz, H-2), 3.80 – 3.61 (1H, m, H-7), 3.58 (2H, s, H-12), 1.89 – 1.51 (9H, m, CH_{2eq}), 1.39 – 0.81 (13H, m, CH_{2ax}); **¹³C NMR (75 MHz, CDCl₃)** δ 170.30 and 168.63 (C-11 and C-1), 138.27 (Ar-C), 134.56 (Ar-C), 129.34 (Ar-C), 128.98 (Ar-C), 128.94 (Ar-C), 128.31 (Ar-C), 127.33 (Ar-C), 127.22 (Ar-C), 57.16 (C-2), 48.73 (C-7), 43.62 (C-12), 32.77 and 32.58 (C-8, C-8'), 25.35 (C-10), 24.68 and 24.54 (C-9, C-9'); **HRMS m/z**: calculated for C₂₂H₂₇N₂O₂: 351.2073, found [M + H]⁺: 351.2076.

4.2.2 U-4CR Enzyme Reactions

4.2.2.1 Activity test reaction of Proteases

N-acetylglycine ethyl ester (20 mg, 0.14 mmol) was dissolved in 25 mM phosphate buffer of pH 7.5 (2 ml) and the solution was stirred at 40 °C. Protease enzyme (350 mg) was added and the mixture was left to stir for 24 hours. TLC was used to monitor the reaction and ascertain if the enzyme was active and had effectively hydrolysed the substrate. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.2.2.2 Activity test reaction of Penicillin G Acylase

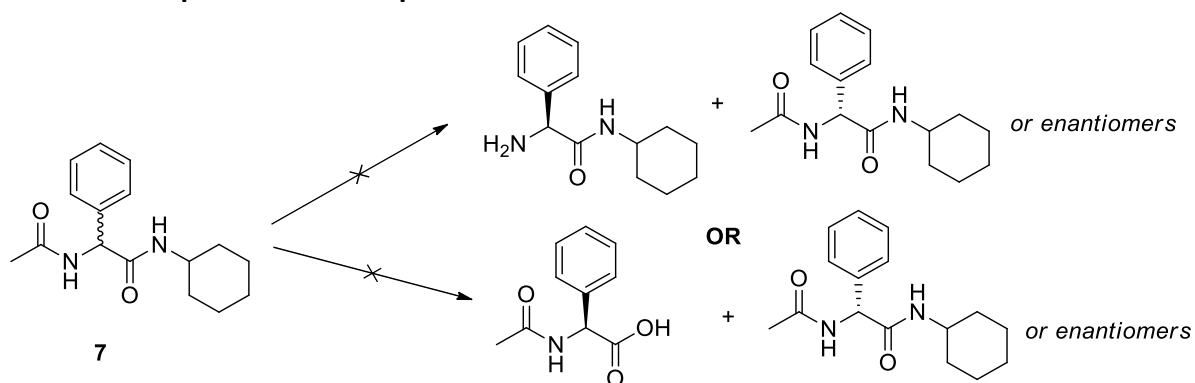
Penicillin G sodium salt (10 mg, 0.03 mmol) was dissolved in 25 mM phosphate buffer of pH 7.5 (1 ml) and the solution was stirred at 40 °C. Penicillin G acylase enzyme (30 μ l) was added and the mixture was left to stir for 24 hours. TLC was used to monitor the reaction and ascertain if the enzyme was active and had effectively hydrolysed the substrate. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

From the six enzymes tested, all exhibited activity against the respective test substrates.

List of active enzymes:

1. Alcalase (FE201)
2. *B. clausii* protease (FE202)
3. *B. lentus* protease (FE203)
4. *Bacillus*. species protease (FE212)
5. *B. licheniformis* protease (FE213)
6. Penicillin G acylase

4.2.2.3 Attempted EKR of compound 7

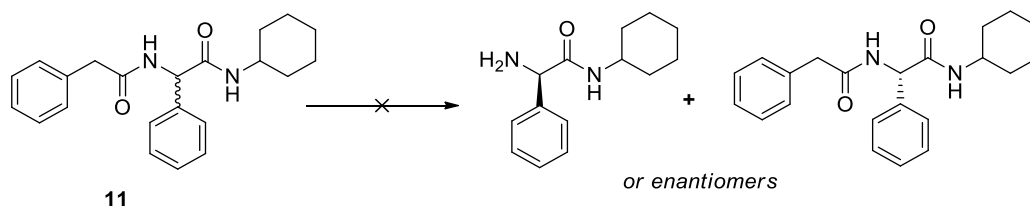


Compound **7** (10 mg, 0.04 mmol) was dissolved in DMSO (1 ml). A portion of this solution (0.2 ml) was then added to 25 mM phosphate buffer of pH 7.5 (1 ml) and the mixture was stirred at 40 °C. Protease enzyme (1200 mg) was added and the reaction was left to stir for 30 d. No reaction was observed from TLC analysis for all active proteases and the experiment was discontinued. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.2.2.4 Attempted EKR of compound 7 in a two-phased system

Compound **7** (10 mg, 0.04 mmol) was dissolved in MBE (0.5 ml). The solution was added to 25 mM phosphate buffer of pH 7.5 (1 ml) and the two-phased mixture was stirred at 40 °C. Protease enzyme (1200 mg) was then added and the reaction was left to stir for 14 d. No reaction was observed from TLC analysis for all active proteases and the experiment was discontinued. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.2.2.5 Attempted EKR of compound of 11



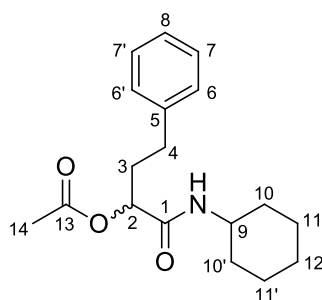
Compound **11** (10 mg, 0.03 mmol) was dissolved in EtOH (1 ml). The solution was then added to 25 mM phosphate buffer of pH 7.5 (9 ml) and the mixture was stirred at 40 °C. Penicillin G acylase enzyme (30 μ l) was added and the reaction was left to stir for 30 d. No reaction was observed from TLC analysis and the experiment was discontinued. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.3 Enzymatic Kinetic Resolution of Passerini Enantiomers

4.3.1 Synthesis of Passerini Enantiomers and Hydrolysis Standards

4.3.1.1 Synthesis of 1-(cyclohexylamino)-1-oxo-4-phenylbutan-2-yl acetate **12**

Hydrocinnamaldehyde (658 μ l, 5.00 mmol), acetic acid (286 μ l, 5.00 mmol) and cyclohexyl isocyanide (622 μ l, 5.00 mmol) were stirred in CH_2Cl_2 (10 ml) at room temperature for 3 d. Excess solvent was removed *in vacuo* and the residue was purified by column chromatography (EtOAc/Hexane 0:1 to 3:7) to yield **12** (0.92 g, 61%) as a white solid, as a racemate.

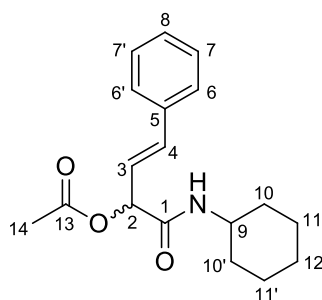


R_f = 0.66 (50% EtOAc/Hexane); **m.p.** 100 – 103 °C; **IR** ($\nu_{\text{max}}/\text{cm}^{-1}$): 3284 (NH), 2936, 2855 (C-H), 1740, 1650 (C=O), 1556 (C=C), 1232 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.36 – 7.08 (5H, m, Ar-H), 5.86 (1H, d, J = 8.0 Hz, NH), 5.13 (1H, t, J = 5.9 Hz, H-2), 3.87 – 3.69 (1H, m, H-9), 2.67 (2H, t, J = 7.9 Hz, H-4), 2.17 (3H, s, H-14), 2.10 (2H, s, H-3), 1.97 – 1.84 and 1.75 – 1.55 (5H, 2xm, $\text{CH}_{2\text{eq}}$), 1.47 – 1.05 (5H, m, $\text{CH}_{2\text{ax}}$); **^{13}C NMR (75 MHz, CDCl_3)** δ 169.64 and 168.46 (C-13 and C-1), 140.85 (C-5), 128.48 and 128.38 (C-7, C-7' and C-6, C-6'), 126.11 (C-8), 73.75 (C-2), 48.02 (C-9), 33.32 (C-3), 31.12 (C-4), 30.91 (C-10, C-10'),

25.49 (C-12), 24.78 (C-11, C-11'), 20.92 (C-14); **HRMS m/z**: calculated for C₁₈H₂₆NO₃: 304.1913, found [M + H]⁺: 304.1908.

4.3.1.2 Synthesis of (*E*)-1-(cyclohexylamino)-1-oxo-4-phenylbut-3-en-2-yl acetate **13**

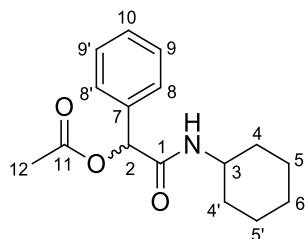
trans-Cinnamaldehyde (630 µl, 5.00 mmol), acetic acid (286 µl, 5.00 mmol) and cyclohexyl isocyanide (622 µl, 5.00 mmol) were stirred in CH₂Cl₂ (10 ml) at room temperature for 3 d. The preparation was completed using the same procedure as described in **4.3.1.1** to yield **13** (0.66 g, 44%) as a white solid, as a racemate.



R_f = 0.64 (50% EtOAc/Hexane), **m.p.** 123 – 125 °C (lit m.p. 132 – 133 °C)⁸³; **IR (ν_{max}/cm⁻¹)**: 3289 (NH), 2934, 2853 (C-H), 1733, 1661 (C=O), 1547 (C=C), 1229 (C-O), 1056 (C-N), 964 (*trans* CH=CH); **¹H NMR (300 MHz, CDCl₃)** δ 7.46 – 7.19 (5H, m, Ar-*H*), 6.73 (1H, dd, *J* = 16.0, 0.9 Hz, *H*-4), 6.27 (1H, dd, *J* = 15.9, 6.8 Hz, *H*-3), 5.89 (1H, d, *J* = 7.9 Hz, NH), 5.71 (1H, dd, *J* = 6.9, 1.2 Hz, *H*-2), 3.89 – 3.69 (1H, m, *H*-9), 2.20 (3H, s, *H*-14), 1.98 – 1.87 and 1.76 – 1.53 (5H, 2xm, CH_{2eq}), 1.47 – 1.28 and 1.27 – 1.05 (5H, 2xm, CH_{2ax}). **¹³C NMR (75 MHz, CDCl₃)** δ 169.18 and 167.04 (C-13 and C-1), 135.72 (C-4), 134.72 (C-5), 128.61 (C-7, C-7'), 128.40 (C-8), 126.83 (C-6, C-6'), 122.73 (C-3), 74.50 (C-2), 48.26 (C-9), 33.02 (C-10, C-10'), 25.48 (C-12), 24.78 (C-11, C-11'), 21.07 (C-14); **HRMS m/z**: calculated for C₁₈H₂₄NO₃: 302.1756, found [M + H]⁺: 302.1759.

4.3.1.3 Synthesis of 2-(cyclohexylamino)-2-oxo-1-phenylethyl acetate **14**

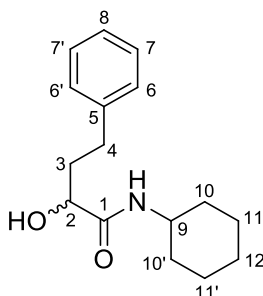
Benzaldehyde (508 µl, 5.00 mmol), acetic acid (286 µl, 5.00 mmol) and cyclohexyl isocyanide (622 µl, 5.00 mmol) were stirred in CH₂Cl₂ (10 ml) at room temperature for 3 d. The preparation was completed using the same procedure as described in **4.3.1.1** to yield **14** (0.79 g, 57%) as a white solid, as a racemate.



R_f = 0.64 (50% EtOAc/Hexane); **m.p.** 123 – 125 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3318 (NH), 2934, 2855 (C-H), 1738, 1660 (C=O), 1543 (C=C), 1235 (C-O); $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.56 – 7.20 (5H, m, Ar-*H*), 6.76 – 6.55 (1H, m, NH), 6.02 (1H, s, *H*-2), 3.88 – 3.60 (1H, m, *H*-3), 2.10 (3H, s, *H*-12), 1.94 – 1.47 (5H, m, $\text{CH}_{2\text{eq}}$), 1.40 – 1.00 (5H, m, $\text{CH}_{2\text{ax}}$). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 169.16 and 167.26 (C-11 and C-1), 135.86 (C-7), 128.89 (Ar-C), 128.70 (Ar-C), 127.43 (Ar-C), 75.54 (C-2), 48.23 (C-3), 32.94 (C-4, C-4'), 25.47 (C-6), 24.75 (C-5, C-5'), 21.03 (C-12); **HRMS** m/z : calculated for $\text{C}_{16}\text{H}_{22}\text{NO}_3$: 276.1600, found $[\text{M} + \text{H}]^+$: 276.1600.⁸²

4.3.1.4 Synthesis of *N*-cyclohexyl-2-hydroxy-4-phenylbutanamide **15**

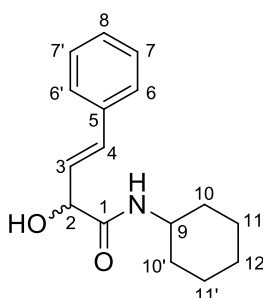
KOH (10 eq., 460 mg, 8.20 mmol) was added to a solution of **12** (250 mg, 0.82 mmol) in MeOH (4 ml). The reaction was left to stir in air at room temperature overnight. H_2O (10 ml) and 6 M HCl (5 ml) were added and the product was extracted with EtOAc (3 x 15 ml). The combined organic layers were dried over MgSO_4 and concentrated *in vacuo* to obtain **15** (0.11 g, 51%) as a cream white solid.



R_f = 0.38 (50% EtOAc/Hexane); **m.p.** 86 - 87 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3284 (NH), 3278 (OH), 2926, 2853 (C-H), 1635 (C=O), 1536 (C=C), 1103 (C-O); $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.41 – 7.03 (5H, m, Ar-*H*), 6.72 (1H, d, J = 8.2 Hz, NH), 4.08 (1H, dd, J = 7.8, 3.5 Hz, *H*-2), 3.82 – 3.62 (1H, m, *H*-9), 2.73 (2H, t, J = 7.8 Hz, *H*-4), 2.18 – 1.51 (7H, m, *H*-3 and $\text{CH}_{2\text{eq}}$), 1.44 – 1.02 (5H, m, $\text{CH}_{2\text{ax}}$). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 173.37 (C-1), 141.45 (C-5), 128.47 and 128.43 (C-7, C-7' and C-6, C-6'), 125.96 (C-8), 71.38 (C-2), 47.96 (C-9), 36.55 (C-3), 32.92 (C-10, C-10'), 31.21 (C-4), 25.46 (C-12), 24.77 (C-11, C-11'); **HRMS** m/z : calculated for $\text{C}_{16}\text{H}_{24}\text{NO}_2$: 262.1807, found $[\text{M} + \text{H}]^+$: 262.1809.⁸⁴

4.3.1.5 Synthesis of (*E*)-*N*-cyclohexyl-2-hydroxy-4-phenylbut-3-enamide **16**

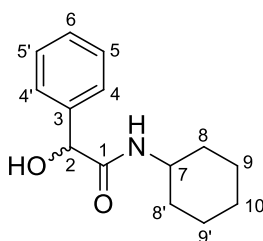
1.5 M H₂SO₄ (3 ml) was added to a suspension of **13** (250 mg, 0.83 mmol) in EtOH (9 ml). The reaction was heated mildly under reflux for 90 min. Excess solvent was removed *in vacuo* and the product extracted with EtOAc (3 x 20 ml). The combined organic layers were washed with H₂O (4 x 20 ml) followed by brine (20 ml) and dried over MgSO₄. Excess solvent was removed *in vacuo* and the residue was purified by column chromatography (EtOAc/Hexane 0:1 to 1:1) to yield **16** (0.16 g, 74%) as a yellow solid.



R_f = 0.30 (50% EtOAc/Hexane); **m.p.** 90 – 93 °C (lit m.p. 79 - 79.5 °C)⁸³; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3395 (NH), 3272 (OH), 2928, 2855 (C-H), 1636 (C=O), 1541 (C=C), 966 (*trans* CH=CH); **¹H NMR** (300 MHz, CDCl₃) δ 7.48 – 7.14 (5H, m, Ar-H), 6.74 (1H, d, J = 15.8 Hz, *H*-4), 6.43 – 6.20 (1H, m, *H*-3), 6.25 (1H, d, J = 7.0 Hz, NH), 4.78 (1H, d, J = 7.0 Hz, *H*-2), 3.85 – 3.68 (1H, m, *H*-9), 1.96 – 1.50 (5H, m, CH_{2eq}), 1.42 – 1.01 (5H, m, CH_{2ax}); **¹³C NMR** (75 MHz, CDCl₃) δ 171.66 (C-1), 135.97 (C-4), 133.47 (C-5), 128.61 (C-7, C-7'), 128.15 (C-8), 126.76 (C-6, C-6'), 126.65 (C-3), 72.75 (C-2), 48.69 (C-9), 32.81 (C-10, C-10'), 25.39 (C-12), 24.72 (C-11, C-11'); **HRMS m/z**: calculated for C₁₆H₂₂NO₂: 260.1651, found [M + H]⁺: 260.1657.

4.3.1.6 Synthesis of *N*-cyclohexyl-2-hydroxy-2-phenylacetamide **17**

KOH (10 eq., 511 mg, 9.10 mmol) was added to a solution of **14** (250 mg, 0.91 mmol) in MeOH (4 ml). The preparation was completed using the same procedure as described in **4.3.1.4** to obtain **17** (0.16 g, 75%) as a cream white solid.



R_f = 0.34 (50% EtOAc/Hexane); **m.p.** 94 – 96 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3335 (NH), 3251 (OH), 2931, 2852 (C-H), 1637 (C=O), 1539 (C=C), 1195 (C-O), 1058 (C-N); **¹H NMR** (300 MHz, CDCl₃) δ 7.43 - 7.21 (5H, m, Ar-H), 5.96 (1H, s, NH), 4.97 (1H, s, *H*-2), 3.81 – 3.57 (1H, m, *H*-7), 1.93 – 1.52 (5H, m, CH_{2eq}), 1.43 – 0.96 (5H, m, CH_{2ax}); **¹³C NMR** (75 MHz, CDCl₃) δ 171.37 (C-1), 139.84 (C-3), 128.69 (C-5, C-5'), 128.40 (C-6),

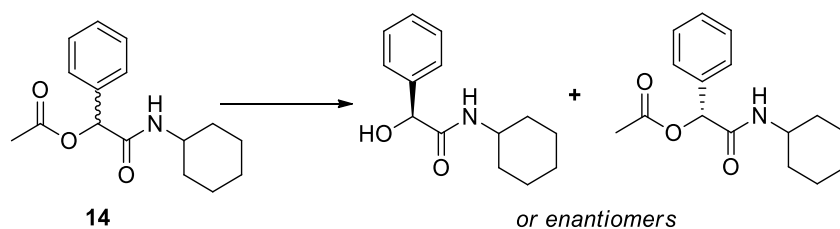
126.81 (C-4, C-4'), 74.06 (C-2), 48.28 (C-7), 32.87 (C-8, C-8'), 25.44 (C-10), 24.68 (C-9, C-9'); **HRMS m/z**: calculated for $C_{14}H_{20}NO_2$: 234.1494, found $[M + H]^+$: 234.1505.⁸⁵

4.3.2 Passerini Enzyme Reactions

4.3.2.1 Activity test reaction of Lipases

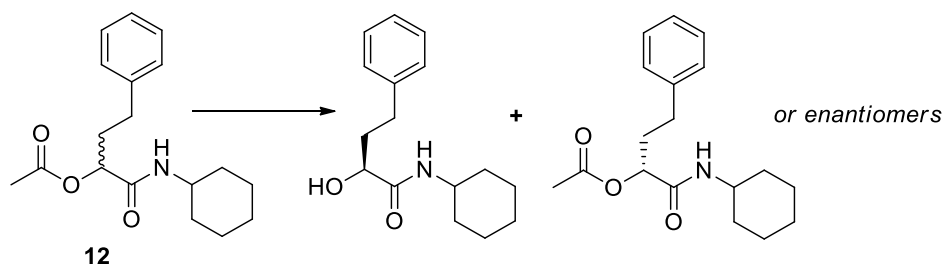
Benzyl acetate (20 mg, 0.13 mmol) was dissolved in MeCN (100 μ l). The solution was added to a mixture of 50 mM phosphate buffer of pH 7.0 (100 μ l) and Lipase 6Y/ 6Z (40 mg). The reaction was then stirred at 40 °C for 3 h. TLC was used to monitor the reaction and ascertain if the enzyme was active and had effectively hydrolysed the substrate. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme. Both Lipase 6Y and 6Z exhibited activity against the test substrate.

4.3.2.2 EKR of compound of 14 with Lipases 6Y and 6Z



Compound **14** (20 mg, 0.07 mmol) was dissolved in MeCN (100 μ l). The solution was added to a mixture of 50 mM phosphate buffer of pH 7.0 (100 μ l) and Lipase 6Y/ 6Z (40 mg). The reaction was then stirred at 40 °C for 3 h. A portion of the reaction mixture was removed at 20 min intervals and the organic layer extracted with EtOAc. The residue was then subjected to chiral HPLC and both enzymes was found to hydrolyse **14** to **17**. The conversion, e.e. and enantioselectivity at each time period of the reaction was determined using HPLC peak area. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.3.2.3 EKR of compound of 12 with Lipase 6Y



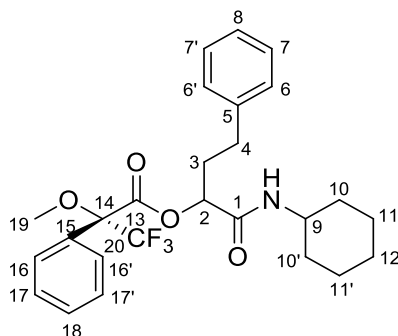
Compound **12** (200 mg, 0.66 mmol) was dissolved in MeCN (5 ml). The solution was added to a mixture of 50 mM phosphate buffer of pH 7.0 (5 ml) and Lipase 6Y (400 mg). The reaction was then stirred at 40 °C for 12 h. The organic layer was extracted with EtOAc. Excess solvent was removed *in vacuo* and the sample was subjected to chiral HPLC with the conversion determined to be 45.7% using HPLC peak area. The residue was purified by column chromatography (EtOAc/Hexane) to yield both starting material and compound **15** (38.0 mg, 22%) as a scalemic mixture. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.3.2.4 EKR of compound of **12** with Lipase 6Z

Compound **12** (200 mg, 0.66 mmol) was dissolved in MeCN (5 ml). The solution was added to a mixture of 50 mM phosphate buffer of pH 7.0 (5 ml) and Lipase 6Z (400 mg). The reaction was then stirred at 40 °C for 12 h. The organic layer was extracted with EtOAc. Excess solvent was removed *in vacuo* and the sample was subjected to chiral HPLC with the conversion determined to be 31.2% using HPLC peak area. The residue was purified by column chromatography (EtOAc/Hexane) to yield both starting material and compound **15** (45.3 mg, 26%) as a scalemic mixture. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.3.2.5 Synthesis of (2*R*)-1-(cyclohexylamino)-1-oxo-4-phenylbutan-2-yl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate **18** from EKR with Lipase 6Y

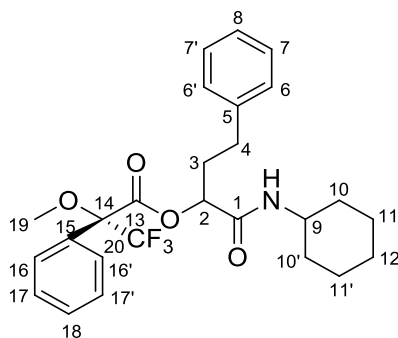
Compound **15** (38.0 mg, 0.15 mmol) as a scalemic mixture obtained from the enzyme reaction, was dissolved in anhydrous CH₂Cl₂ (4 ml) and the solution was stirred for 10 min. (*R*)-MTPA (3.2 eq., 111 mg, 0.48 mmol) and DCC (2.4 eq., 74.2 mg, 0.36 mmol) were added to CH₂Cl₂ (6 ml) and the mixture was added to the stirring solution. DMAP (0.4 eq., 7.50 mg, 0.06 mmol) was then added and the reaction was left to stir for 90 min. The product was then extracted with CH₂Cl₂ (3 x 20 ml) and the combined organic layers dried over Na₂SO₄. Solvent was removed *in vacuo* and the residue was purified by column chromatography (EtOAc/Hexane 0:1 to 1:1) to yield **18** (47.7 mg, 67%), a thick clear liquid, as a mixture of diastereomers. The e.e. of the reaction was determined from the relative integration of ¹⁹F NMR.



R_f = 0.92 (50% EtOAc/Hexane); **e.e.**: 79.4%; **E**: 14.7; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3306 (NH), 2932, 2855 (C-H), 1753, 1660 (C=O), 1167 (C-O), 1017 (C-F); **^1H NMR (300 MHz, CDCl_3)** δ 7.63 – 7.35 (5H, m, Ar-H), 7.33 – 7.01 (5H, m, Ar-H), 5.80 (1H, d, J = 8.1 Hz, NH), 5.41 – 5.26 (1H, m, H-2), 3.84 – 3.66 (1H, m, H-9), 3.55 (3H, s, H-19), 2.64 – 2.49 (2H, m, H-4), 2.31 – 2.11 (2H, m, H-3), 1.87 – 1.44 and 1.42 – 0.95 (17H, 2xm, cyclohexyl CH_2); **^{13}C NMR (75 MHz, CDCl_3)** δ 167.31 and 165.29 (C-13 and C-1), 140.36 (Ar-C), 131.54 (Ar-C), 130.05 (Ar-C), 128.83 (Ar-C), 128.54 (Ar-C), 128.37 (Ar-C), 127.25 (Ar-C), 126.26 (Ar-C), 125.30 (C-20), 85.00 and 84.63 (C-14), 75.34 (C-2), 55.40 (C-19), 48.03 (C-9), 33.67, 32.85, 32.77 and 30.50 (C-10, C-10', C-4 and C-3), 25.40 (C-12), 24.57 (C-11, C-11'); **^{19}F NMR (471 MHz, CDCl_3)** δ -70.66 (1F, s, CF_3), -70.82 (9F, s, CF_3).

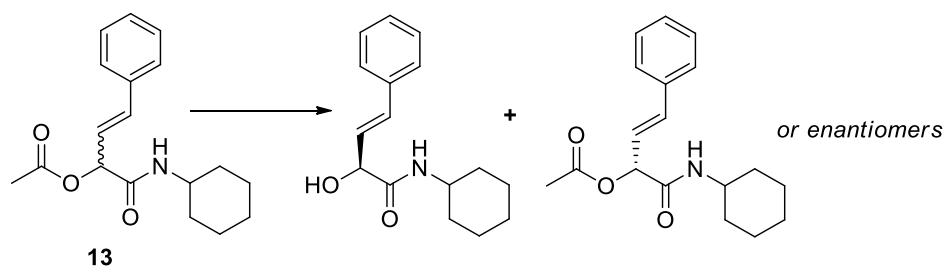
4.3.2.6 Synthesis of (2R)-1-(cyclohexylamino)-1-oxo-4-phenylbutan-2-yl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate **18** from EKR with Lipase 6Z

Compound **15** (45.3 mg, 0.17 mmol) as a scalemic mixture obtained from the enzyme reaction, was dissolved in anhydrous CH_2Cl_2 (4 ml) and the solution was stirred for 10 min. (*R*)-MTPA (3.2 eq., 126 mg, 0.54 mmol) and DCC (2.4 eq., 84.1 mg, 0.41 mmol) were added to CH_2Cl_2 (6 ml) and the mixture was added to the stirring solution. DMAP (0.4 eq., 8.50 mg, 0.07 mmol) was then added and the reaction was left to stir for 90 min. The preparation was completed using the same procedure as described in **4.3.2.4** to yield **18** (63.7 mg, 78%), a thick clear liquid, as a mixture of diastereomers. The e.e. of the reaction was determined from the relative integration of ^{19}F NMR.



e.e.: 92.7%; **E**: 40.6; **^{19}F NMR (471 MHz, CDCl_3)** δ -70.66 (1F, s, CF_3), -70.82 (10F, s, CF_3).

4.3.2.7 EKR of compound **13** with Lipase 6Y



Compound **13** (200 mg, 0.66 mmol) was dissolved in MeCN (5 ml). The solution was added to a mixture of 50 mM phosphate buffer of pH 7.0 (5 ml) and Lipase 6Y (400 mg). The reaction was then stirred at 40 °C for 20 h. The organic layer was extracted with EtOAc. Excess solvent was removed *in vacuo* and the sample was subjected to chiral HPLC with the conversion determined to be 21.5% using HPLC peak area. The residue was purified by column chromatography (EtOAc/Hexane) to yield both starting material and compound **16** (23.9 mg, 14%) as a scalemic mixture. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.3.2.8 EKR of compound **13** with Lipase 6Z

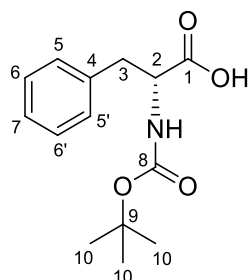
Compound **13** (200 mg, 0.66 mmol) was dissolved in MeCN (5 ml). The solution was added to a mixture of 50 mM phosphate buffer of pH 7.0 (5 ml) and Lipase 6Z (400 mg). The reaction was then stirred at 40 °C for 20 h. The organic layer was extracted with EtOAc. Excess solvent was removed *in vacuo* and the sample was subjected to chiral HPLC with the conversion determined to be 24.4% using HPLC peak area. The residue was purified by column chromatography (EtOAc/Hexane) to yield both starting material and compound **16** (82.1 mg, 48%) as a scalemic mixture. A control reaction containing no enzyme was performed to establish that hydrolysis failed to occur in the absence of enzyme.

4.4 Diastereoselectivity of the Passerini reaction

4.4.1 Synthesis of *N*-Boc-D-phenylalanine **19**

D-phenylalanine (5.00 g, 30.3 mmol) was added to 1,4-dioxane (44 ml) and the mixture was vigorously stirred for 20 min. 1 M NaOH (26 ml) was added and the reaction was stirred for a further 20 min at 0 °C. Boc anhydride (1.75 eq., 12.2 ml, 53.0 mmol) was then added in small amounts and the mixture was left to stir overnight at room temperature. The reaction was acidified to a pH of 2 by dropwise addition of 1 M KHSO₄. The solvent was removed *in vacuo* and H₂O (100 ml) was added. The product was then extracted with EtOAc (3 x 100 ml) and the combined organic layers washed with brine (200

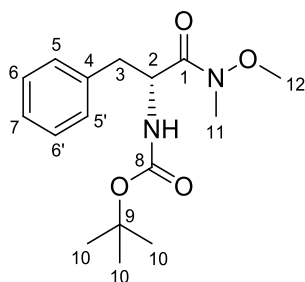
ml) and dried over MgSO_4 . Solvent was removed *in vacuo* and the residue was purified by column chromatography (2% $\text{MeOH}/\text{CHCl}_3$) to yield *N*-Boc-D-phenylalanine **19** (5.37 g, 67%) as a clear glutinous oil.



R_f = 0.29 (5% $\text{MeOH}/\text{CHCl}_3$); IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3306 (NH), 3245 (OH), 2978, 2937 (C-H), 1709, 1644 (C=O), 1155 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 10.96 (1H, s, OH), 7.43 – 7.10 (5H, m, Ar-H), 6.66 (0.35H, d, J = 7.7 Hz, NH), 5.20 (0.49H, d, J = 8.1 Hz, NH), 4.71 – 4.52 (0.51H, m, H-2), 4.48 – 4.24 (0.37H, m, H-2), 3.36 – 2.69 (2H, m, H-3), 1.60 - 1.08 (9H, m, H-10); ^{13}C NMR (75 MHz, CDCl_3) δ 175.72 (C-1), 156.76 and 155.47 (C-8), 136.50 and 136.06 (C-4), 129.43 (C-6, C-6'), 128.48 (C-5, C-5'), 126.91 (C-7), 81.65 and 80.17 (C-9), 56.25 and 54.31 (C-2), 38.84 and 37.84 (C-3), 28.27 and 27.97 (C-10).

4.4.2 Synthesis of (*R*)-*tert*-butyl (1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate **20**

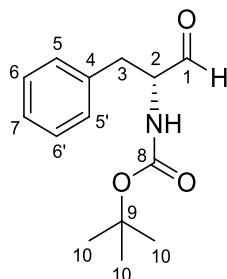
N-Boc-D-phenylalanine **19** (4.00 g, 15.1 mmol) was dissolved in CH_2Cl_2 (40 ml). CDI (1.2 eq., 2.93 g, 18.1 mmol) was added to the solution in small portions, observing effervescence at each addition. The mixture was stirred for 45 min after which *N,O*-dimethylhydroxylamine hydrochloride (1.2 eq., 1.77 g, 18.1 mmol) was added. The reaction was left to stir in air at room temperature for 48 h. CH_2Cl_2 (90 ml) was added and the reaction mixture was washed with H_2O (90 ml) followed by 2 M NaOH (90 ml) and 1 M HCl (90 ml). The organic layer was dried over MgSO_4 and the solvent removed *in vacuo* to afford the amide **20** (4.19 g, 90%) as a thick colourless oil that was used without further purification.



R_f = 0.57 (50% EtOAc/Hexane); IR ($\nu_{\max}/\text{cm}^{-1}$): 3316 (NH), 2976, 2934 (C-H), 1700, 1656 (C=O), 1163 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.34 – 7.10 (5H, m, Ar-H), 5.16 (1H, d, J = 9.2 Hz, NH), 4.95 (1H, d, J = 5.8 Hz, H-2), 3.65 (3H, s, H-12), 3.16 (3H, s, H-11), 3.05 (1H, dd, J = 13.6, 6.1 Hz, H-3), 2.96 – 2.70 (1H, m, H-3), 1.38 (9H, s, H-10); ^{13}C NMR (75 MHz, CDCl_3) δ 172.30 (C-1), 155.16 (C-8), 136.59 (C-4), 129.45 (C-6, C-6'), 128.33 (C-5, C-5'), 126.74 (C-7), 79.57 (C-9), 61.54 (C-12), 51.52 (C-2), 38.88 (C-3), 32.06 (C-11), 28.31 (C-10).

4.4.3 Synthesis of (*R*)-*tert*-butyl (1-oxo-3-phenylpropan-2-yl)carbamate **21**

The Weinreb amide **20** (1.45 g, 4.70 mmol) was added to dry THF (80 ml) in a two-necked round-bottomed flask. The solution was cooled to 0 °C and LiAlH_4 (1.5 eq., 0.27 g, 7.05 mmol) was added in one portion to the stirring solution under N_2 . The reaction was left to stir for 90 min. KHSO_4 (1.5 eq., 0.96 g, 7.05 mmol) was then added slowly with vigorous stirring for 30 min. EtOAc (90 ml) was added and the reaction mixture was washed with 1 M HCl (90 ml) followed by a saturated solution of NaHCO_3 (90 ml) and brine (90 ml). The organic layer was dried over MgSO_4 and concentrated *in vacuo* to obtain **21** (1.01 g, 86%) as a white solid.

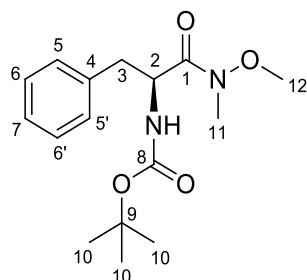


R_f = 0.78 (50% EtOAc/Hexane); $[\alpha]_D^{20} +40.6$ (c 0.50, MeOH); *m.p.* 82 – 83 °C; IR ($\nu_{\max}/\text{cm}^{-1}$): 3367 (NH), 2980, 2845 (C-H), 1729, 1687 (C=O), 1519 (C=C), 1165 (C-O), 1054 (C-N); ^1H NMR (300 MHz, CDCl_3) δ 9.63 (1H, s, H-1), 7.36 – 7.13 (5H, m, Ar-H), 5.14 – 4.92 (1H, m, NH), 4.52 – 4.33 (1H, m, H-2), 3.12 (2H, d, J = 6.4 Hz, H-3), 1.44 (9H, s, H-10); ^{13}C NMR (75 MHz, CDCl_3) δ 199.37 (C-1), 155.35 (C-8), 135.77 (C-4), 129.33 (C-6, C-6'), 128.76 (C-5, C-5'), 127.08 (C-7), 80.21 (C-9), 60.79 (C-2), 35.51 (C-3), 28.28 (C-10).

4.4.4 Synthesis of (*S*)-*tert*-butyl (1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate **22**

N-Boc-L-phenylalanine (4.00 g, 15.1 mmol) was dissolved in CH_2Cl_2 (40 ml). CDI (1.2 eq., 2.93 g, 18.1 mmol) was added to the solution in small portions, observing effervescence at each addition. The mixture was stirred for 45 min after which *N,O*-dimethylhydroxylamine hydrochloride (1.2 eq., 1.77 g,

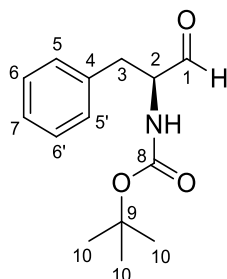
18.1 mmol) was added. The preparation was completed using the same procedure as described in 4.4.2 to afford the amide **22** (4.31 g, 93%) as a thick colourless oil that was used without further purification.



R_f = 0.56 (50% EtOAc/Hexane); IR ($\nu_{\max}/\text{cm}^{-1}$): 3321 (NH), 2976, 2936 (C-H), 1708, 1654 (C=O), 1164 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.34 – 7.10 (5H, m, Ar-H), 5.16 (1H, d, J = 7.8 Hz, NH), 4.95 (1H, d, J = 6.5 Hz, H-2), 3.65 (3H, s, H-12), 3.16 (3H, s, H-11), 3.05 (1H, dd, J = 13.5, 6.0 Hz, H-3), 2.96 – 2.69 (1H, m, H-3), 1.38 (9H, s, H-10); ^{13}C NMR (75 MHz, CDCl_3) δ 172.32 (C-1), 155.17 (C-8), 136.59 (C-4), 129.45 (C-6, C-6'), 128.33 (C-5, C-5'), 126.75 (C-7), 79.58 (C-9), 61.55 (C-12), 51.53 (C-2), 38.86 (C-3), 32.07 (C-11), 28.31 (C-10).

4.4.5 Synthesis of (S)-tert-butyl (1-oxo-3-phenylpropan-2-yl)carbamate **23**

The Weinreb amide **22** (4.00 g, 13.0 mmol) was added to dry THF (80 ml) in a two-necked round-bottomed flask. The solution was cooled to 0 °C and LiAlH_4 (1.5 eq., 0.74 g, 19.5 mmol) was added in one portion to the stirring solution under N_2 . The reaction was left to stir for 90 min. KHSO_4 (1.5 eq., 2.66 g, 19.5 mmol) was then added slowly with vigorous stirring for 30 min. The preparation was completed using the same procedure as described in 4.4.3 to obtain **23** (2.52 g, 78%) as a white solid.



R_f = 0.78 (50% EtOAc/Hexane); $[\alpha]_{\text{D}}^{20}$ -41.4 (c 0.50, MeOH); *m.p.* 81 – 83 °C (lit *m.p.* 86 °C)⁹¹; IR ($\nu_{\max}/\text{cm}^{-1}$): 3366 (NH), 2982, 2846 (C-H), 1730, 1687 (C=O), 1519 (C=C), 1164 (C-O), 1054 (C-N); ^1H NMR (300 MHz, CDCl_3) δ 9.54 (1H, s, H-1), 7.38 – 7.12 (5H, m, Ar-H), 5.49 – 5.38 (1H, m, NH), 4.39 – 4.22 (1H, m, H-2), 3.21 – 3.04 (1H, m, H-3), 3.02 – 2.92 (1H, m, H-3), 1.40 (9H, s, H-10); ^{13}C NMR (75

MHz, CDCl₃) δ 199.62 (C-1), 155.48 (C-8), 136.27 (C-4), 129.29 (C-6, C-6'), 128.58 (C-5, C-5'), 126.84 (C-7), 79.85 (C-9), 60.78 (C-2), 35.06 (C-3), 28.22 (C-10).

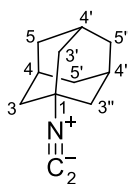
4.4.6 Synthesis of 1-isocyanoadamantane **24**

4.4.6.1 Formylation of adamantylamine

Adamantylamine (0.50 g, 3.31 mmol) was added to acetic anhydride (10 ml), followed by formic acid (10 eq., 1.25 ml, 33.1 mmol). The mixture was stirred under N₂ at room temperature overnight. The reaction was then cooled in ice and quenched using small successive amounts of solid Na₂CO₃. After effervescence had subsided, brine (100 ml) was added and the product extracted with EtOAc (2 x 100 ml). The combined organic layers were dried over MgSO₄ and concentrated *in vacuo* to obtain adamantyl formamide (0.55 g crude) as a white solid.

4.4.6.2 Dehydration of adamantyl formamide

Anhydrous CH₂Cl₂ (50 ml) was added under N₂ to crude adamantyl formamide (0.55 g, $\approx$ 3.07 mmol). Et₃N (18 eq., 7.71 ml, 55.3 mmol) was then added gradually and the solution was cooled to 0 °C. POCl₃ (3 eq., 0.86 ml, 9.21 mmol) was added dropwise and the reaction was stirred for 30 min. The mixture was then warmed to room temperature and left to stir overnight under N₂. The reaction was cooled in ice and quenched using small portions of solid Na₂CO₃. After effervescence had ceased, brine (100 ml) was added and the product extracted with CH₂Cl₂ (100 ml). The organic layer was dried over MgSO₄ and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc/Hexane) yielded **24** (0.43 g, 81%) as a pale yellow solid with a pungent odour.

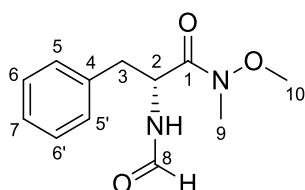


R_f = 0.87 (50% EtOAc/Hexane) visualised with iodine; **m.p.** 184 – 187 °C (lit m.p. 188 - 193 °C)⁹³; **¹H NMR (300 MHz, CDCl₃)** δ 2.15 – 2.06 (3H, m, *H*-4, *H*-4', *H*-4''), 2.03 – 2.02 (6H, m, *H*-3, *H*-3', *H*-3''), 1.68 – 1.66 (6H, m, *H*-5, *H*-5', *H*-5''); **¹³C NMR (75 MHz, CDCl₃)** δ 151.81 (t, J_{C-N} = 5.12 Hz, C-2), 54.21 (t, J_{C-N} = 6.9 Hz, C-1), 43.60 (C-3, C-3', C-3''), 35.50 (C-5, C-5', C-5''), 28.74 (C-4, C-4', C-4'').

4.4.7 Synthesis of (*R*)-2-isocyano-*N*-methoxy-*N*-methyl-3-phenylpropanamide **26**

4.4.7.1 Synthesis of (*R*)-2-formamido-*N*-methoxy-*N*-methyl-3-phenylpropanamide **25**

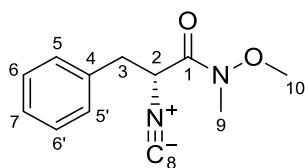
Compound **20** (4.29 g, 13.9 mmol) was added to acetic anhydride (10 ml), followed by formic acid (35 ml). The mixture was stirred under N₂ at room temperature for 10 min. TFA (5 eq., 5.32 ml, 69.5 mmol) was then added dropwise and the reaction was left to stir overnight. The mixture was cooled in ice and quenched using small successive amounts of solid Na₂CO₃. After effervescence had subsided, brine (200 ml) was added and the product extracted with EtOAc (2 x 100 ml). The combined organic layers were dried over MgSO₄ and concentrated *in vacuo* to obtain **25** (3.38 g crude) as a thick yellowish oil that was used without further purification.



R_f = 0.03 (5% MeOH/CH₂Cl₂); IR ($\nu_{\max}/\text{cm}^{-1}$): 3290 (NH), 2972, 2865 (C-H), 1642 (C=O); ¹H NMR (300 MHz, CDCl₃) δ 8.13 (1H, s, *H*-8), 7.38 – 7.12 (5H, m, Ar-*H*), 6.46 (1H, d, *J* = 7.2 Hz, NH), 5.35 (1H, dd, *J* = 14.4, 7.6 Hz, *H*-2), 3.69 (3H, s, *H*-10), 3.19 and 3.12 (4H, s overlapped with dd, *J* = 13.8, 6.0 Hz, *H*-3 and *H*-9), 2.97 (1H, dd, *J* = 13.6, 7.0 Hz, *H*-3); ¹³C NMR (75 MHz, CDCl₃) δ 171.26 (C-8), 160.52 (C-1), 135.99 (C-4), 129.45 (C-6, C-6'), 128.44 (C-5, C-5'), 127.02 (C-7), 61.66 (C-10), 48.91 (C-2), 38.21 (C-3), 32.14 (C-9); HRMS *m/z*: calculated for C₁₂H₁₇N₂O₃: 237.1239, found [M + H]⁺: 237.1242.

4.4.7.2 Dehydration of (*R*)-2-formamido-*N*-methoxy-*N*-methyl-3-phenylpropanamide

Compound **25** (3.38 g, $\approx$ 14.3 mmol) was dissolved in CH₂Cl₂ (60 ml) followed by the addition of PPh₃ (1.5 eq., 5.64 g, 21.5 mmol), CCl₄ (1.5 eq., 2.08 ml, 21.5 mmol), and DIPEA (1.5 eq., 3.75 ml, 21.5 mmol). The mixture was stirred under N₂ at room temperature overnight. Excess solvent was removed *in vacuo* and the residue was purified by column chromatography (EtOAc/Hexane 1:9 to 3:7) to yield **26** (0.53 g, 17%) as a light brown oil.

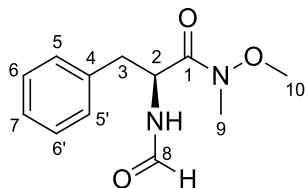


R_f = 0.56 (50% EtOAc/Hexane); $[\alpha_D]^{20}_{589}$ -6.45 (c 2.00, CHCl₃); IR ($\nu_{\max}/\text{cm}^{-1}$): 2967, 2844 (C-H), 2145 (C≡N), 1653 (C=O); ¹H NMR (300 MHz, CDCl₃) δ 7.41 – 7.23 (5H, m, Ar-H), 4.85 – 4.74 (1H, m, H-2), 3.66 (3H, s, H-10), 3.30 – 3.23 and 3.21 (4H, m overlapped with s, H-3 and H-9), 3.19 – 3.05 (1H, m, H-3). ¹³C NMR (75 MHz, CDCl₃) δ 165.83 (C-1), 159.24 (C-8), 135.24 (C-4), 129.31 (C-6, C-6'), 128.63 (C-7), 127.55 (C-5, C-5'), 61.66 (C-10), 55.51 (C-2), 38.70 (C-3), 32.50 (C-9); HRMS m/z : calculated for C₁₂H₁₅N₂O₂: 219.1134, found [M + H]⁺: 219.1144.

4.4.8 Synthesis of (S)-2-isocyano-N-methoxy-N-methyl-3-phenylpropanamide 28

4.4.8.1 Synthesis of (S)-2-formamido-N-methoxy-N-methyl-3-phenylpropanamide 27

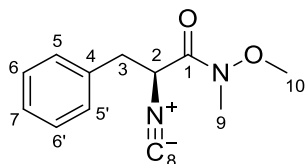
Compound **22** (5.33 g, 17.3 mmol) was added to acetic anhydride (10 ml), followed by formic acid (35 ml). The mixture was stirred under N₂ at room temperature for 10 min. TFA (5 eq., 6.62 ml, 86.5 mmol) was then added dropwise and the reaction was left to stir overnight. The preparation was completed using the same procedure as described in 4.4.7.1 to yield **27** (1.13 g, 28%) as a thick yellow oil.



R_f = 0.03 (5% MeOH/CH₂Cl₂); IR ($\nu_{\max}/\text{cm}^{-1}$): 3298 (NH), 2940 (C-H), 1643 (C=O); ¹H NMR (300 MHz, CDCl₃) δ 8.07 (1H, s, H-8), 7.50 (1H, d, J = 8.0 Hz, NH), 7.31 – 7.12 (5H, m, Ar-H), 5.42 – 5.24 (1H, m, H-2), 3.66 (3H, s, H-10), 3.14 and 3.10 – 3.02 (4H, s overlapped with m, H-9 and H-3), 2.98 – 2.85 (1H, m, H-3); ¹³C NMR (75 MHz, CDCl₃) δ 171.57 (C-8), 161.13 (C-1), 136.36 (C-4), 129.38 (C-6, C-6'), 128.33 (C-5, C-5'), 126.85 (C-7), 61.55 (C-10), 48.99 (C-2), 38.04 (C-3), 32.01 (C-9); HRMS m/z : calculated for C₁₂H₁₇N₂O₃: 237.1239, found [M + H]⁺: 237.1246.

4.4.8.2 Dehydration of (S)-2-formamido-N-methoxy-N-methyl-3-phenylpropanamide

Compound **27** (1.13 g, 4.78 mmol) was dissolved in CH₂Cl₂ (60 ml) followed by the addition of PPh₃ (1.5 eq., 1.88 g, 7.17 mmol), CCl₄ (1.5 eq., 694 μ l, 7.17 mmol), and DIPEA (1.5 eq., 1.25 ml, 7.17 mmol). The preparation was completed using the same procedure as described in 4.4.7.2 to yield **28** (0.74 g, 71%) as a light brown oil.

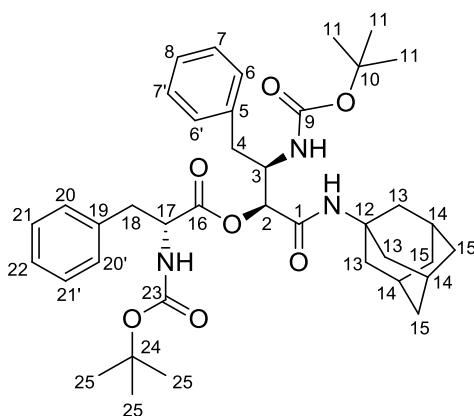


R_f = 0.56 (50% EtOAc/Hexane); $[\alpha_D]^{20}_{589} +3.55$ (c 2.00, CHCl_3); IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 2973, 2844 (C-H), 2144 ($\text{C}\equiv\text{N}$), 1676 ($\text{C}=\text{O}$); ^1H NMR (300 MHz, CDCl_3) δ 7.43 – 7.13 (5H, m, Ar-*H*), 4.88 – 4.71 (1H, m, *H*-2), 3.62 (3H, s, *H*-10), 3.29 – 3.19 and 3.17 (4H, m overlapped with s, *H*-3 and *H*-9), 3.13 – 3.00 (1H, m, *H*-3); ^{13}C NMR (75 MHz, CDCl_3) δ 165.78 (C-1), 159.23 (C-8), 135.23 (C-4), 129.30 (C-6, C-6'), 128.69 (C-5, C-5'), 127.51 (C-7), 61.67 (C-10), 55.52 (C-2), 38.67 (C-3), 32.48 (C-9); HRMS m/z : calculated for $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_2$: 219.1134, found $[\text{M} + \text{H}]^+$: 219.1132.

4.4.9 Synthesis of (2*R*)-(3*R*)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 29

Adamantyl isocyanide (161 mg, 1.00 mmol), *N*-Boc-D-phenylalaninal (249 mg, 1.00 mmol) and *N*-Boc-D-phenylalanine (265 mg, 1.00 mmol) were stirred in CH_2Cl_2 (3 ml) at room temperature for 5 d. Excess solvent was removed *in vacuo* and the residue was purified by column chromatography (EtOAc/Hexane) to yield **29** (0.62 g, 92%) as a (2.29 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **29A** and the minor **29B**.

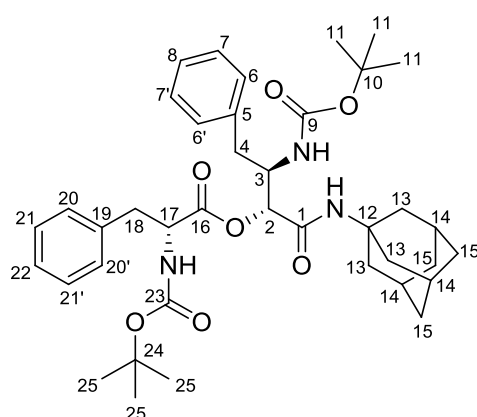
(2*R*)-(2*S*,3*R*)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 29A



R_f = 0.86 (50% EtOAc/Hexane); *m.p.* 92 – 94 °C; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3394, 3354 (NH), 2974, 2911 (C-H), 1749, 1713, 1669 ($\text{C}=\text{O}$), 1542, 1497 ($\text{C}=\text{C}$), 1160 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.42 – 7.03 (13H, m, Ar-*H*), 6.30 (1H, s, NH), 5.22 (1H, d, J = 9.5 Hz, NH), 5.14 – 4.97 (1H, m, *H*-2), 4.96 – 4.86 (1H, m, NH), 4.35 – 4.25 and 4.24 – 4.11 (2H, 2xm, *H*-3 and *H*-17), 3.22 – 2.90 and 2.65 – 2.41 (4H, 2xm, *H*-4

and *H*-18), 2.14 – 1.94 (9H, m, *H*-13 and *H*-14), 1.66 (6H, s, *H*-15), 1.54 – 1.24 (18H, m, *H*-11 and *H*-25); ¹³C NMR (75 MHz, CDCl₃) δ 171.09 and 166.91 (C-16 and C-1), 155.53 and 154.78 (C-23 and C-9), 137.53 (Ar-C), 135.62 (Ar-C), 129.42 (Ar-C), 129.13 (Ar-C), 128.98 (Ar-C), 128.29 (Ar-C), 127.52 (Ar-C), 126.40 (Ar-C), 80.53 and 79.19 (C-24 and C-10), 74.32 (C-2), 55.55, 52.84 and 52.53 (C-17, C-12 and C-3), 41.19 (C-13), 37.79 and 37.33 (C-18 and C-4), 36.40 (C-15), 30.91 (C-14), 29.48 and 28.27 (C-25 and C-11); HRMS *m/z*: calculated for C₃₉H₅₄N₃O₇: 676.3962, found [M + H]⁺: 676.3967.

(2*R*)-(2*R*,3*R*)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 29B



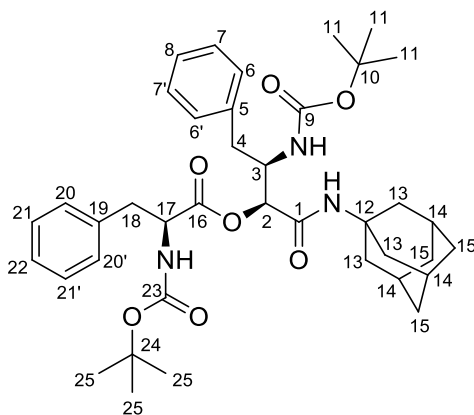
R_f = 0.86 (50% EtOAc/Hexane); *m.p.* 86 – 88 °C; IR (*v*_{max}/cm⁻¹): 3326 (NH), 2975, 2910 (C-H), 1752, 1694 (C=O), 1497 (C=C), 1164 (C-O); ¹H NMR (300 MHz, CDCl₃) δ 7.41 – 7.08 (11H, m, Ar-*H*), 6.32 and 6.01 (1H, 2xs, NH), 5.37 – 4.78 (3H, m, 2xNH and *H*-2), 4.67 – 4.05 (2H, m, *H*-3 and *H*-17), 3.37 – 2.37 (4H, m, *H*-4 and *H*-18), 2.09 – 1.98 (9H, m, *H*-13 and *H*-14), 1.71 – 1.62 (6H, m, *H*-15), 1.45 – 1.26 (18H, m, *H*-11 and *H*-25); ¹³C NMR (75 MHz, CDCl₃) δ 170.74 and 166.09 (C-16 and C-1), 155.51 and 154.76 (C-23 and C-9), 137.13 (Ar-C), 135.93 (Ar-C), 129.40 (Ar-C), 129.11 (Ar-C), 128.97 (Ar-C), 128.27 (Ar-C), 127.21 (Ar-C), 126.55 (Ar-C), 80.55 and 79.51 (C-24 and C-10), 75.74 (C-2), 54.75, 52.82 and 52.52 (C-17, C-12 and C-3), 41.18 (C-13), 37.77 and 37.30 (C-18 and C-4), 36.32 (C-15), 30.90 (C-14), 29.46 and 28.26 (C-25 and C-11); HRMS *m/z*: calculated for C₃₉H₅₄N₃O₇: 676.3962, found [M + H]⁺: 676.3967.

4.4.10 Synthesis of (2*S*)-(3*R*)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 30

Adamantyl isocyanide (242 mg, 1.50 mmol), *N*-Boc-D-phenylalaninal (374 mg, 1.50 mmol) and *N*-Boc-L-phenylalanine (398 mg, 1.50 mmol) were stirred in CH₂Cl₂ (3 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **30** (0.69 g, 68%)

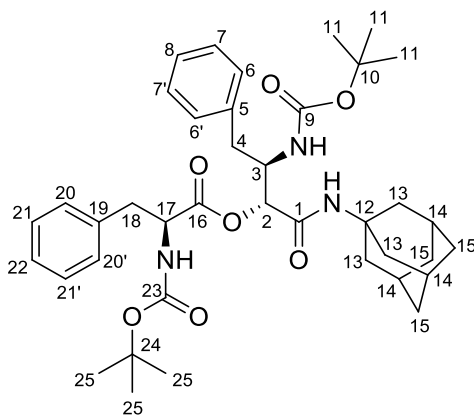
as a (2.47 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **30A** and the minor **30B**.

(2S)-(2S,3R)-1-(adamantan-1-ylamino)-3-((tert-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 30A



R_f = 0.86 (50% EtOAc/Hexane); **m.p.** 181 – 183 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3273 (NH), 2977, 2912 (C-H), 1747, 1720, 1689, 1643 (C=O), 1547, 1492 (C=C), 1164 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.47 – 7.05 (10H, m, Ar-H), 6.11 (1H, s, NH), 5.21 – 4.98 (2H, m, NH and H-2), 4.90 – 4.74 (1H, m, NH), 4.61 – 4.42 and 4.40 – 4.20 (2H, 2xm, H-3 and H-17), 3.27 – 2.94 and 2.86 – 2.51 (4H, 2xm, H-4 and H-18), 2.13 – 1.92 (9H, m, H-13 and H-14), 1.66 (6H, s, H-15), 1.51 – 1.19 (18H, m, H-11 and H-25); **^{13}C NMR (75 MHz, CDCl_3)** δ 170.28 and 166.62 (C-16 and C-1), 155.70 and 154.87 (C-23 and C-9), 137.42 (Ar-C), 135.85 (Ar-C), 129.34 (Ar-C), 129.18 (Ar-C), 128.96 (Ar-C), 128.45 (Ar-C), 127.40 (Ar-C), 126.54 (Ar-C), 80.51 and 79.43 (C-24 and C-10), 74.76 (C-2), 54.77, 52.85 and 52.55 (C-17, C-12 and C-3), 41.25 (C-13), 38.07 and 37.40 (C-18 and C-4), 36.35 (C-15), 30.90 (C-14), 29.46 and 28.36 (C-25 and C-11); **HRMS m/z**: calculated for $\text{C}_{39}\text{H}_{54}\text{N}_3\text{O}_7$: 676.3962, found $[\text{M} + \text{H}]^+$: 676.3968.

(2S)-(2R,3R)-1-(adamantan-1-ylamino)-3-((tert-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 30B

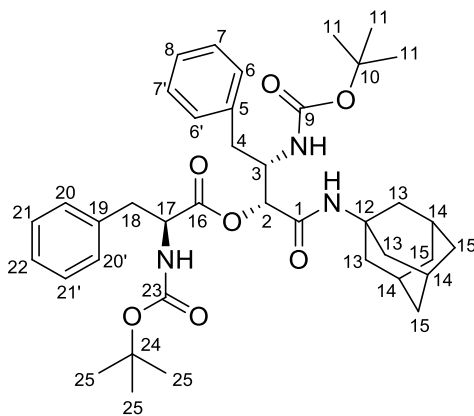


R_f = 0.86 (50% EtOAc/Hexane); **m.p.** 185 – 187 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3271 (NH), 2973, 2921 (C-H), 1748, 1720, 1689, 1644 (C=O), 1541, 1493 (C=C), 1163, 1057 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.54 – 6.98 (10H, m, Ar-*H*), 6.30 (1H, s, NH), 5.40 – 5.22 and 5.17 – 4.96 (2H, 2xm, NH and *H*-2), 4.50 – 4.37, 4.36 – 4.20 and 4.20 – 4.07 (3H, 3xm, NH, *H*-3 and *H*-17), 3.21 – 2.96 and 2.82 – 2.60 (4H, 2xm, *H*-4 and *H*-18), 2.10 – 1.94 (9H, m, *H*-13 and *H*-14), 1.67 (6H, s, *H*-15), 1.50 – 1.30 (18H, m, *H*-11 and *H*-25); **^{13}C NMR (126 MHz, CDCl_3)** δ 170.28 and 166.60 (C-16 and C-1), 155.67 and 154.87 (C-23 and C-9), 137.39 (Ar-C), 135.80 (Ar-C), 129.33 (Ar-C), 129.17 (Ar-C), 128.95 (Ar-C), 128.45 (Ar-C), 127.38 (Ar-C), 126.54 (Ar-C), 80.49 and 79.39 (C-24 and C-10), 74.73 (C-2), 54.71, 52.83 and 52.55 (C-17, C-12 and C-3), 41.23 (C-13), 38.03 and 37.33 (C-18 and C-4), 36.34 (C-15), 30.92 (C-14), 29.44 and 28.35 (C-25 and C-11); **HRMS m/z** : calculated for $\text{C}_{39}\text{H}_{54}\text{N}_3\text{O}_7$: 676.3962, found $[\text{M} + \text{H}]^+$: 676.3968.

4.4.11 Synthesis of (2S)-(3S)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **31**

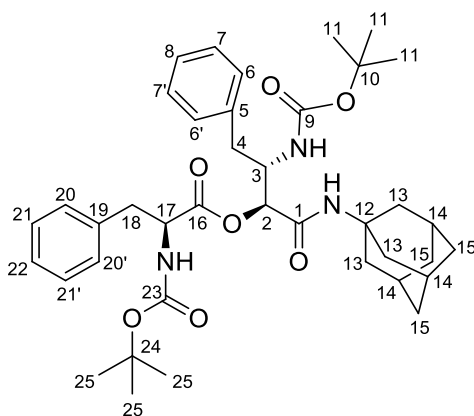
Adamantyl isocyanide (81 mg, 0.50 mmol), *N*-Boc-L-phenylalaninal (125 mg, 0.50 mmol) and *N*-Boc-L-phenylalanine (133 mg, 0.50 mmol) were stirred in CH_2Cl_2 (3 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **31** (0.19 g, 56%) as a (2.26 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **31A** and the minor **31B**.

(2S)-(2R,3S)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **31A**



R_f = 0.86 (50% EtOAc/Hexane); **m.p.** 79 – 80 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 2973, 2922 (C-H), 1751, 1695, 1655 (C=O), 1535, 1497 (C=C), 1165, 1054 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.40 – 7.10 (19H, m, Ar-H), 6.30 (1H, s, NH), 5.23 (1H, d, J = 9.0 Hz, NH), 5.16 – 5.01 (1H, m, H-2), 5.01 – 4.85 (1H, m, NH), 4.38 – 4.23 and 4.24 – 4.09 (2H, 2xm, H-3 and H-17), 3.14 – 2.92 and 2.66 – 2.47 (4H, 2xm, H-4 and H-18), 2.07 – 2.01 (9H, m, H-13 and H-14), 1.66 – 1.59 (6H, m, H-15), 1.44 – 1.34 (18H, m, H-11 and H-25); **^{13}C NMR (126 MHz, CDCl_3)** δ 171.06 and 166.83 (C-16 and C-1), 155.48 and 154.71 (C-23 and C-9), 137.51 (Ar-C), 135.46 (Ar-C), 129.40 (Ar-C), 129.09 (Ar-C), 128.99 (Ar-C), 128.28 (Ar-C), 127.54 (Ar-C), 126.39 (Ar-C), 80.60 and 79.18 (C-24 and C-10), 74.26 (C-2), 55.47, 52.81 and 52.55 (C-17, C-12 and C-3), 41.18 (C-13), 37.74 and 37.28 (C-18 and C-4), 36.38 (C-15), 30.93 (C-14), 29.45 and 28.25 (C-25 and C-11); **HRMS m/z** : calculated for $\text{C}_{39}\text{H}_{54}\text{N}_3\text{O}_7$: 676.3962, found $[\text{M} + \text{H}]^+$: 676.3955.

(2S)-(2S,3S)-1-(adamantan-1-ylamino)-3-((tert-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 31B



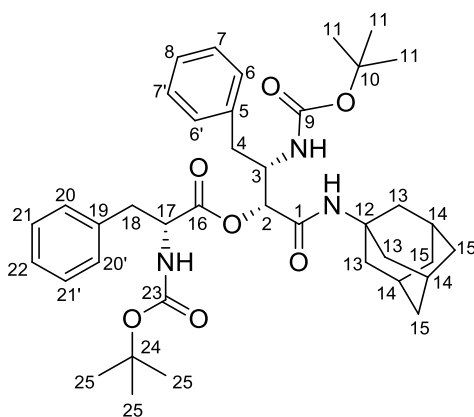
R_f = 0.86 (50% EtOAc/Hexane); **m.p.** 80 – 82 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 2981, 2922 (C-H), 1751, 1690, 1651 (C=O), 1531, 1498 (C=C), 1163, 1055 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.39 – 7.09 (13H, m, Ar-H), 6.29 and 6.00 (1H, 2xs, NH), 5.31 – 5.00 (2H, m, NH and H-2), 4.98 – 4.76 (1H, m, NH), 4.61 – 4.10 (2H, m, H-3 and H-17), 3.24 – 2.72 and 2.63 – 2.49 (4H, 2xm, H-4 and H-18), 2.09 – 1.98 (9H, m, H-13 and

H-14), 1.69 – 1.65 (6H, m, *H*-15), 1.46 – 1.32 (18H, m, *H*-11 and *H*-25); ^{13}C NMR (126 MHz, CDCl_3) δ 170.73 and 166.06 (C-16 and C-1), 155.49 and 154.76 (C-23 and C-9), 137.85 (Ar-C), 135.90 (Ar-C), 129.40 (Ar-C), 129.10 (Ar-C), 128.98 (Ar-C), 128.28 (Ar-C), 127.20 (Ar-C), 126.57 (Ar-C), 80.57 and 79.18 (C-24 and C-10), 74.27 (C-2), 55.46, 52.81 and 52.42 (C-17, C-12 and C-3), 41.30 (C-13), 37.75 and 37.29 (C-18 and C-4), 36.31 (C-15), 30.93 (C-14), 29.45 and 28.26 (C-25 and C-11); HRMS m/z : calculated for $\text{C}_{39}\text{H}_{54}\text{N}_3\text{O}_7$: 676.3962, found $[\text{M} + \text{H}]^+$: 676.3955.

4.4.12 Synthesis of (2*R*)-(3*S*)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **32**

Adamantyl isocyanide (161 mg, 1.00 mmol), *N*-Boc-L-phenylalaninal (249 mg, 1.00 mmol) and *N*-Boc-D-phenylalanine (265 mg, 1.00 mmol) were stirred in CH_2Cl_2 (3 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **32** to yield (0.62 g, 92%) as a (2.08 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **32A** and the minor **32B**.

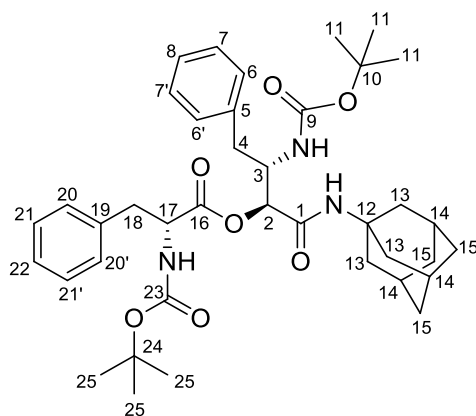
(2*R*)-(2*R*,3*S*)-1-(adamantan-1-ylamino)-3-((*tert*-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **32A**



R_f = 0.86 (50% EtOAc/Hexane); *m.p.* 192 – 195 °C; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3275 (NH), 2967, 2922 (C-H), 1747, 1721, 1689, 1644 (C=O), 1538, 1491 (C=C), 1163, 1056 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.43 – 7.09 (10H, m, Ar-*H*), 6.10 (1H, s, NH), 5.21 – 4.96 (2H, m, NH and *H*-2), 4.89 – 4.74 (1H, m, NH), 4.60 – 4.43 and 4.39 – 4.21 (2H, 2xm, *H*-3 and *H*-17), 3.27 – 2.94 and 2.87 – 2.57 (4H, 2xm, *H*-4 and *H*-18), 2.10 – 1.95 (9H, m, *H*-13 and *H*-14), 1.66 (6H, s, *H*-15), 1.53 – 1.22 (18H, m, *H*-11 and *H*-25); ^{13}C NMR (75 MHz, CDCl_3) δ 170.26 and 166.59 (C-16 and C-1), 155.65 and 154.84 (C-23 and C-9), 137.41 (Ar-C), 135.79 (Ar-C), 129.33 (Ar-C), 129.16 (Ar-C), 128.95 (Ar-C), 128.44 (Ar-C), 127.40 (Ar-C), 126.53 (Ar-C), 80.51

and 79.42 (C-24 and C-10), 74.73 (C-2), 54.73, 52.82 and 52.54 (C-17, C-12 and C-3), 41.23 (C-13), 38.02 and 37.35 (C-18 and C-4), 36.33 (C-15), 30.91 (C-14), 29.44 and 28.35 (C-25 and C-11); **HRMS m/z**: calculated for C₃₉H₅₄N₃O₇: 676.3962, found [M + H]⁺: 676.3973.

(2R)-(2S,3S)-1-(adamantan-1-ylamino)-3-((tert-butoxycarbonyl)amino)-1-oxo-4-phenylbutan-2-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 32B



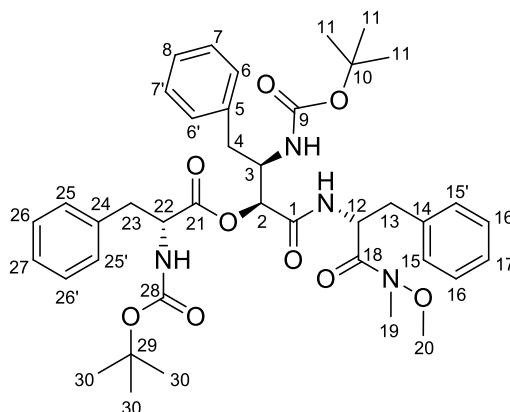
R_f = 0.86 (50% EtOAc/Hexane); **m.p.** 102 – 104 °C; **IR** (ν_{max} /cm⁻¹): 3342 (NH), 2973, 2921 (C-H), 1760, 1692, 1654 (C=O), 1540, 1497 (C=C), 1163, 1056 (C-O); **¹H NMR** (300 MHz, CDCl₃) δ 7.42 – 7.03 (12H, m, Ar-H), 6.34 and 6.10 (1H, 2xs, NH), 5.40 – 5.23 and 5.16 – 5.01 (2H, 2xm, H-2 and NH), 4.57 – 4.36, 4.36 – 4.24 and 4.22 – 4.06 (3H, 3xm, H-3, H-17 and NH), 3.22 – 2.94 and 2.83 – 2.58 (4H, 2xm, H-4 and H-18), 2.04 – 2.00 (9H, m, H-13 and H-14), 1.67 (6H, s, H-15), 1.49 – 1.29 (18H, m, H-11 and H-25); **¹³C NMR** (75 MHz, CDCl₃) δ 171.51 and 166.11 (C-16 and C-1), 155.77 and 154.99 (C-23 and C-9), 137.60 (Ar-C), 135.79 (Ar-C), 129.39 (Ar-C), 129.14 (Ar-C), 128.96 (Ar-C), 128.44 (Ar-C), 127.48 (Ar-C), 126.44 (Ar-C), 80.53 and 79.40 (C-24 and C-10), 75.22 (C-2), 55.86, 53.01 and 52.41 (C-17, C-12 and C-3), 41.22 (C-13), 38.01 and 37.76 (C-18 and C-4), 36.38 (C-15), 30.91 (C-14), 29.46 and 28.26 (C-25 and C-11); **HRMS m/z**: calculated for C₃₉H₅₄N₃O₇: 676.3962, found [M + H]⁺: 676.3973.

4.4.13 Synthesis of (2R)-(5R,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 33

D-phenylalanine isocyanide (100 mg, 0.46 mmol), *N*-Boc-D-phenylalaninal (115 mg, 0.46 mmol) and *N*-Boc-D-phenylalanine (122 mg, 0.46 mmol) were stirred in CH₂Cl₂ (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **33** (0.14 g,

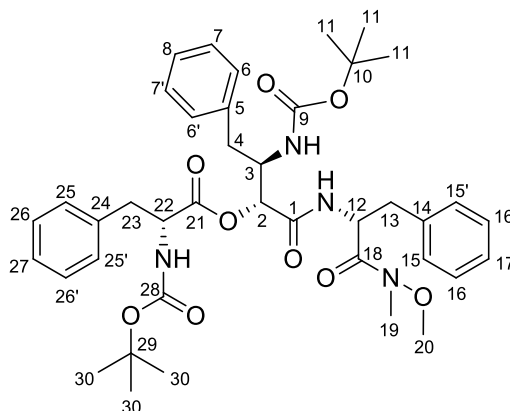
42%) as a (1.97 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **33A** and the minor **33B**.

(2R)-(5R,8S,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 33A



R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 136 – 139 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3358 (NH), 2981, 2936 (C-H), 1756, 1691, 1660 (C=O), 1528 (C=C), 1166 (C-O). **^1H NMR (300 MHz, CDCl_3)** δ 7.38 – 7.09 (17H, m, Ar-H), 5.35 – 5.11 (2H, m, H-2 and H-12), 5.10 – 4.99 (1H, m, NH), 4.99 – 4.87 (1H, m, NH), 4.52 – 4.32 and 4.30 – 4.10 (2H, 2xm, H-22 and H-3), 3.62 (3H, s, H-20), 3.25 – 2.83 and 2.64 – 2.43 (10H, 2xm, NH, H-4, H-13, H-19 and H-23), 1.52 – 1.25 (18H, m, H-11 and H-30); **^{13}C NMR (75 MHz, CDCl_3)** δ 171.20, 170.81 and 167.43 (C-21, C-18 and C-1), 155.45 and 154.78 (C-28 and C-9), 137.39 (Ar-C), 136.48 (Ar-C), 135.61 (Ar-C), 129.44 (Ar-C), 129.39 (Ar-C), 129.14 (Ar-C), 128.91 (Ar-C), 128.39 (Ar-C), 128.34 (Ar-C), 127.41 (Ar-C), 126.83 (Ar-C), 126.43 (Ar-C), 80.63 and 79.27 (C-29 and C-10), 73.97 (C-2), 61.56 (C-20), 55.22, 52.90 and 50.23 (C-22, C-12 and C-3), 37.99, 37.71 and 37.40 (C-23, C-13 and C-4), 32.08 (C-19), 28.32 (C-30 and C-11); **HRMS m/z**: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3810.

(2R)-(5R,8R,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 33B

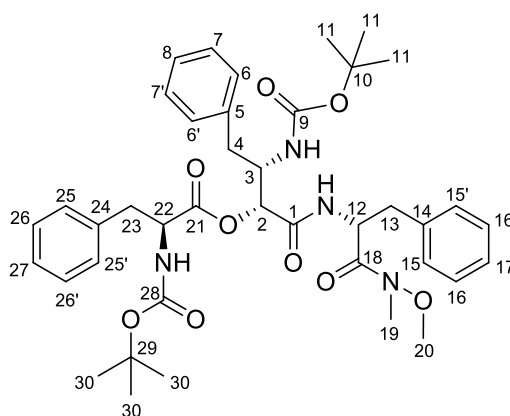


R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 83 – 84 °C; IR ($\nu_{\max}/\text{cm}^{-1}$): 3352 (NH), 2981, 2940 (C-H), 1756, 1689, 1659 (C=O), 1516, 1497 (C=C), 1164 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.46 – 6.99 (18H, m, Ar-*H*), 5.38 – 4.89 and 4.72 – 4.15 (6H, 2xm, 2xNH, *H*-2, *H*-3, *H*-12 and *H*-22), 3.76 – 3.56 (3H, m, *H*-20), 3.29 – 2.83 and 2.75 – 2.45 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.47 – 1.26 (18H, m, *H*-11 and *H*-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.16, 170.66 and 167.37 (C-21, C-18 and C-1), 155.57 and 154.83 (C-28 and C-9), 137.40 (Ar-C), 136.42 (Ar-C), 135.69 (Ar-C), 129.46 (Ar-C), 129.39 (Ar-C), 129.18 (Ar-C), 128.86 (Ar-C), 128.46 (Ar-C), 128.33 (Ar-C), 127.30 (Ar-C), 126.93 (Ar-C), 126.41 (Ar-C), 80.56 and 79.39 (C-29 and C-10), 74.19 (C-2), 61.56 (C-20), 55.14, 52.91 and 50.60 (C-22, C-12 and C-3), 37.93, 37.81 and 37.52 (C-23, C-13 and C-4), 32.09 (C-19), 28.35 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3810.

4.4.14 Synthesis of (2S)-(5R,9S)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate **34**

D-phenylalanine isocyanide (100 mg, 0.46 mmol), *N*-Boc-L-phenylalaninal (115 mg, 0.46 mmol) and *N*-Boc-L-phenylalanine (122 mg, 0.46 mmol) were stirred in CH_2Cl_2 (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **34** (0.17 g, 50%) as a (2.46 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **34A** and the minor **34B**.

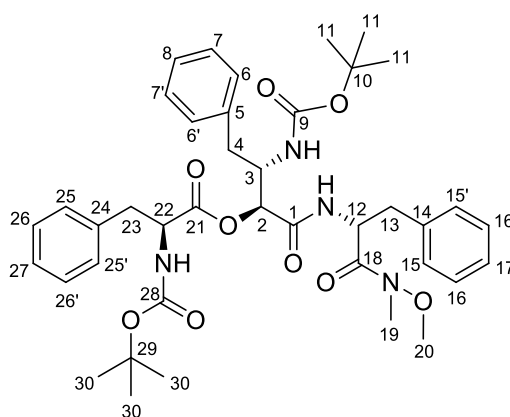
(2S)-(5R,8R,9S)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate **34A**



R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 89 – 90 °C; IR ($\nu_{\max}/\text{cm}^{-1}$): 3349 (NH), 2972, 2940 (C-H), 1755, 1689, 1656 (C=O), 1527, 1497 (C=C), 1164 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.46 – 6.99 (17H, m, Ar-*H*), 5.48 – 4.89 and 4.69 – 4.15 (6H, 2xm, 2xNH, *H*-2, *H*-3, *H*-12 and *H*-22), 3.76 – 3.56 (3H, m, *H*-20),

3.29 – 2.83 and 2.75 – 2.45 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.47 – 1.26 (18H, m, *H*-11 and *H*-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.15, 170.70 and 167.00 (C-21, C-18 and C-1), 155.43 and 154.74 (C-28 and C-9), 137.30 (Ar-C), 136.41 (Ar-C), 135.82 (Ar-C), 129.46 (Ar-C), 129.40 (Ar-C), 129.18 (Ar-C), 128.86 (Ar-C), 128.46 (Ar-C), 128.33 (Ar-C), 127.21 (Ar-C), 126.89 (Ar-C), 126.38 (Ar-C), 80.57 and 79.38 (C-29 and C-10), 74.04 (C-2), 61.58 (C-20), 55.15, 52.81 and 50.58 (C-22, C-12 and C-3), 38.03, 37.68 and 37.36 (C-23, C-13 and C-4), 32.10 (C-19), 28.35 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3814.

(2S)-(5R,8S,9S)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 34B

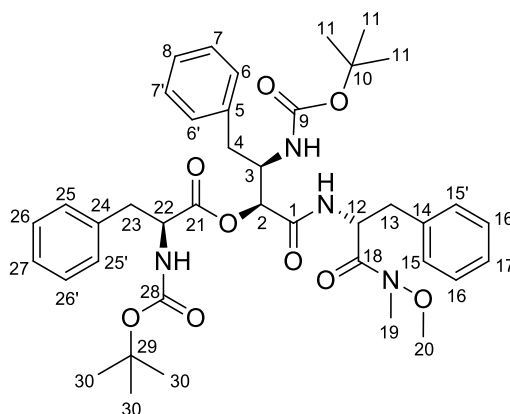


R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 108 – 109 °C; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3358 (NH), 2968, 2937 (C-H), 1755, 1691, 1656 (C=O), 1526, 1497 (C=C), 1166 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.38 – 7.09 (17H, m, Ar-*H*), 5.35 – 5.11 (2H, m, *H*-2 and *H*-12), 5.11 – 5.00 (1H, m, NH), 4.99 – 4.87 (1H, m, NH), 4.52 – 4.32 and 4.30 – 4.10 (2H, 2xm, *H*-22 and *H*-3), 3.62 (3H, s, *H*-20), 3.25 – 2.83 and 2.64 – 2.43 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.52 – 1.25 (18H, m, *H*-11 and *H*-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.16, 170.66 and 167.37 (C-21, C-18 and C-1), 155.57 and 154.83 (C-28 and C-9), 137.40 (Ar-C), 136.42 (Ar-C), 135.69 (Ar-C), 129.46 (Ar-C), 129.39 (Ar-C), 129.18 (Ar-C), 128.86 (Ar-C), 128.46 (Ar-C), 128.33 (Ar-C), 127.30 (Ar-C), 126.93 (Ar-C), 126.41 (Ar-C), 80.56 and 79.39 (C-29 and C-10), 74.19 (C-2), 61.56 (C-20), 55.14, 52.91 and 50.60 (C-22, C-12 and C-3), 37.93, 37.81 and 37.52 (C-23, C-13 and C-4), 32.09 (C-19), 28.35 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3814.

4.4.15 Synthesis of (2S)-(5R,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 35

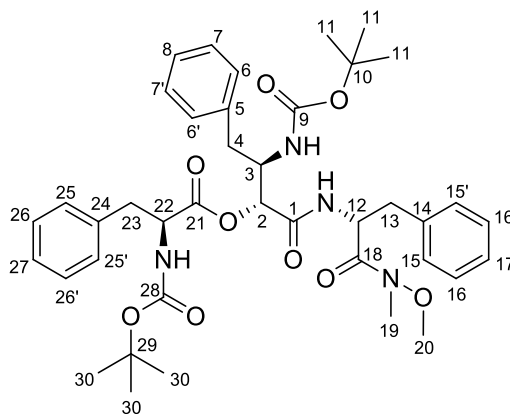
D-phenylalanine isocyanide (100 mg, 0.46 mmol), *N*-Boc-D-phenylalaninal (115 mg, 0.46 mmol) and *N*-Boc-L-phenylalanine (122 mg, 0.46 mmol) were stirred in CH₂Cl₂ (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **35** (0.17 g, 50%) as a (1.80 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **35A** and the minor **35B**.

(2S)-(5R,8S,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 35A



R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 73 – 76 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3309 (NH), 2980, 2937 (C-H), 1746, 1693, 1658 (C=O), 1497, 1455 (C=C), 1161 (C-O); **¹H NMR** (300 MHz, CDCl₃) δ 7.43 – 7.05 (17H, m, Ar-H), 5.31 – 5.03 (2H, m, *H*-2 and *H*-12), 4.98 – 4.77 (2H, m, 2xNH), 4.56 – 4.40 and 4.40 – 4.21 (2H, 2xm, *H*-22 and *H*-3), 3.66 (3H, s, *H*-20), 3.21 – 2.84 and 2.79 – 2.59 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.53 – 1.19 (18H, m, *H*-11 and *H*-30); **¹³C NMR** (75 MHz, CDCl₃) δ 171.04, 170.42 and 167.50 (C-21, C-18 and C-1), 155.62 and 154.90 (C-28 and C-9), 137.34 (Ar-C), 136.41 (Ar-C), 135.96 (Ar-C), 129.47 (Ar-C), 129.35 (Ar-C), 129.19 (Ar-C), 128.89 (Ar-C), 128.46 (Ar-C), 128.41 (Ar-C), 127.26 (Ar-C), 126.89 (Ar-C), 126.52 (Ar-C), 80.66 and 79.43 (C-29 and C-10), 74.46 (C-2), 61.54 (C-20), 54.62, 52.89 and 50.69 (C-22, C-12 and C-3), 37.85, 37.69 and 37.40 (C-23, C-13 and C-4), 32.16 (C-19), 28.36 and 28.28 (C-30 and C-11); **HRMS m/z**: calculated for C₄₀H₅₃N₄O₉: 733.3813, found [M + H]⁺: 733.3807.

(2S)-(5R,8R,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 35B

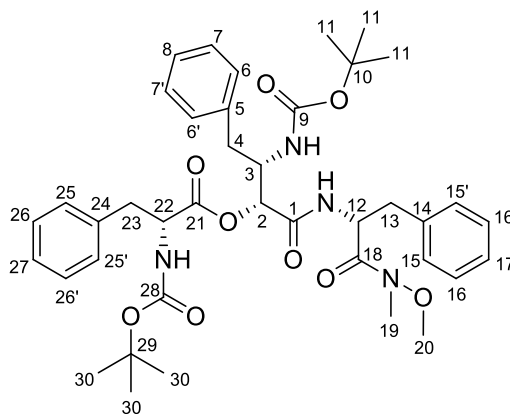


R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 67 – 70 °C; IR ($\nu_{\max}/\text{cm}^{-1}$): 3322 (NH), 2979, 2937 (C-H), 1756, 1689, 1660 (C=O), 1497, 1455 (C=C), 1161 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.45 – 7.02 (18H, m, Ar-H), 5.47 – 4.77 and 4.61 – 4.12 (6H, 2xm, 2xNH, H-2, H-3, H-12 and H-22), 3.77 – 3.58 (3H, m, H-20), 3.23 – 2.83, 2.82 – 2.59 and 2.58 – 2.42 (10H, 3xm, NH, H-4, H-13, H-19 and H-23), 1.54 – 1.19 (18H, m, H-11 and H-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.09, 170.94 and 167.39 (C-21, C-18 and C-1), 155.18 and 154.39 (C-28 and C-9), 137.36 (Ar-C), 136.66 (Ar-C), 136.07 (Ar-C), 129.47 (Ar-C), 129.37 (Ar-C), 129.17 (Ar-C), 128.88 (Ar-C), 128.46 (Ar-C), 128.27 (Ar-C), 127.26 (Ar-C), 126.95 (Ar-C), 126.41 (Ar-C), 80.47 and 79.44 (C-29 and C-10), 75.03 (C-2), 61.57 (C-20), 54.67, 52.61 and 50.28 (C-22, C-12 and C-3), 38.04, 37.85 and 37.38 (C-23, C-13 and C-4), 32.13 (C-19), 28.33 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3807.

4.4.16 Synthesis of (2*R*)-(5*R*,9*S*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **36**

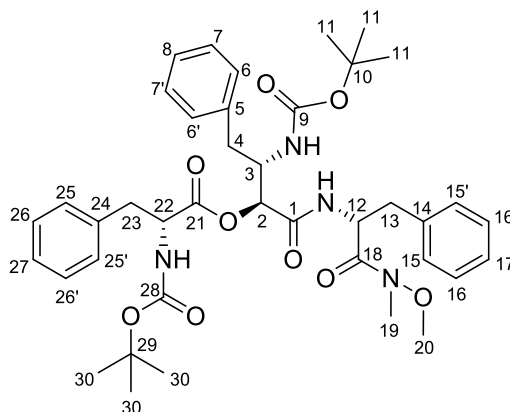
D-phenylalanine isocyanide (100 mg, 0.46 mmol), *N*-Boc-L-phenylalaninal (115 mg, 0.46 mmol) and *N*-Boc-D-phenylalanine (122 mg, 0.46 mmol) were stirred in CH_2Cl_2 (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **36** (0.27 g, 80%) as a (1.94 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **36A** and the minor **36B**.

(2*R*)-(5*R*,8*R*,9*S*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **36A**



R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 83 – 85 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3313 (NH), 2974, 2938 (C-H), 1753, 1695, 1656 (C=O), 1497, 1455 (C=C), 1163 (C-O); **^1H NMR** (300 MHz, CDCl_3) δ 7.45 – 6.99 (17H, m, Ar-H), 5.39 – 4.86 and 4.70 – 4.12 (6H, 2xm, 2xNH, H-2, H-3, H-12 and H-22), 3.81 – 3.55 (3H, m, H-20), 3.31 – 2.76 and 2.75 – 2.46 (10H, 2xm, NH, H-4, H-13, H-19 and H-23), 1.50 – 1.21 (18H, m, H-11 and H-30); **^{13}C NMR** (75 MHz, CDCl_3) δ 171.09, 170.89 and 167.37 (C-21, C-18 and C-1), 155.18 and 154.38 (C-28 and C-9), 137.34 (Ar-C), 136.04 (Ar-C), 135.97 (Ar-C), 129.47 (Ar-C), 129.37 (Ar-C), 129.17 (Ar-C), 128.88 (Ar-C), 128.41 (Ar-C), 128.27 (Ar-C), 127.24 (Ar-C), 126.95 (Ar-C), 126.41 (Ar-C), 80.50 and 79.40 (C-29 and C-10), 75.09 (C-2), 61.51 (C-20), 54.73, 52.56 and 50.25 (C-22, C-12 and C-3), 37.86, 37.72 and 37.35 (C-23, C-13 and C-4), 32.13 (C-19), 28.33 (C-30 and C-11); **HRMS m/z**: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3824.

(2R)-(5R,8S,9S)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate 36B



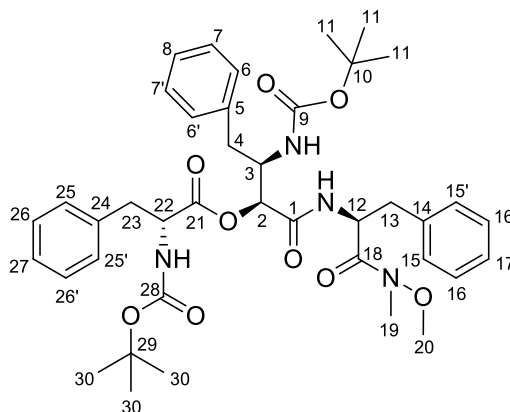
R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 72 – 75 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3312 (NH), 2973, 2938 (C-H), 1753, 1692, 1656 (C=O), 1497, 1455 (C=C), 1163 (C-O); **^1H NMR** (300 MHz, CDCl_3) δ 7.46 – 7.01 (18H, m, Ar-H), 5.37 – 4.89 and 4.71 – 4.15 (6H, 2xm, 2xNH, H-2, H-3, H-12 and H-22), 3.76 – 3.56 (3H, m, H-20), 3.30 – 2.83 and 2.76 – 2.55 (10H, 2xm, NH, H-4, H-13, H-19 and H-23), 1.47 – 1.26 (18H, m, H-11 and

H-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.15, 170.91 and 166.73 (C-21, C-18 and C-1), 155.37 and 155.15 (C-28 and C-9), 137.37 (Ar-C), 136.09 (Ar-C), 135.95 (Ar-C), 129.47 (Ar-C), 129.36 (Ar-C), 129.16 (Ar-C), 128.79 (Ar-C), 128.44 (Ar-C), 128.26 (Ar-C), 127.23 (Ar-C), 126.93 (Ar-C), 126.40 (Ar-C), 80.47 and 79.45 (C-29 and C-10), 75.05 (C-2), 61.57 (C-20), 55.46, 52.66 and 50.29 (C-22, C-12 and C-3), 38.01, 37.86 and 35.96 (C-23, C-13 and C-4), 32.18 (C-19), 28.32 (C-30 and C-11); HRMS m/z : calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3824.

4.4.17 Synthesis of (2*R*)-(5*S*,9*R*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **37**

L-phenylalanine isocyanide (125 mg, 0.57 mmol), *N*-Boc-D-phenylalaninal (142 mg, 0.57 mmol) and *N*-Boc-D-phenylalanine (151 mg, 0.57 mmol) were stirred in CH_2Cl_2 (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **37** (0.37 g, 89%) as a (1.90 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **37A** and the minor **37B**.

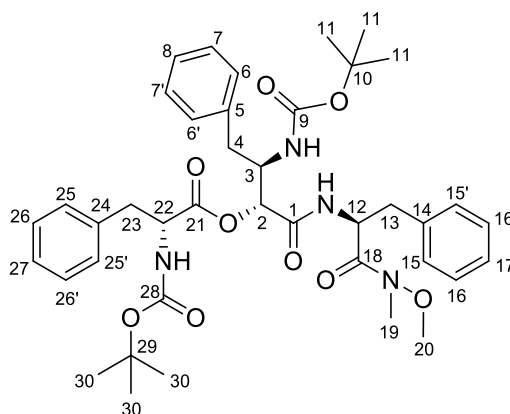
(2*R*)-(5*S*,8*S*,9*R*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate **37A**



R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 104 – 107 °C; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3354 (NH), 2973, 2938 (C-H), 1756, 1690, 1656 (C=O), 1514, 1497 (C=C), 1165 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.38 – 7.11 (18H, m, Ar-*H*), 6.96 – 6.53 (1H, m, NH), 5.32 – 5.09 (2H, m, *H*-2 and *H*-12), 4.99 – 4.83 (1H, m, NH), 4.66 – 4.53 and 4.43 – 4.24 (2H, 2xm, *H*-22 and *H*-3), 3.61 (3H, s, *H*-20), 3.24 – 2.89 and 2.78 – 2.41 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.51 – 1.21 (18H, m, *H*-11 and *H*-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.63, 170.66 and 167.56 (C-21, C-18 and C-1), 155.44 and 154.84 (C-28 and C-9), 137.32 (Ar-C), 136.57 (Ar-C), 135.84 (Ar-C), 129.45 (Ar-C), 129.40 (Ar-C), 129.18 (Ar-C), 128.86 (Ar-C), 128.46 (Ar-C), 128.32 (Ar-C), 127.24 (Ar-C), 127.08 (Ar-C), 126.37 (Ar-C), 80.57 and 79.29 (C-29 and C-10), 74.13 (C-2), 61.41 (C-

20), 55.14, 52.72 and 50.59 (C-22, C-12 and C-3), 37.85, 37.78 and 37.42 (C-23, C-13 and C-4), 32.02 (C-19), 28.32 (C-30 and C-11). **HRMS m/z** : calculated for $C_{40}H_{53}N_4O_9$: 733.3813, found $[M + H]^+$: 733.3822.

(2*R*)-(5*S*,8*R*,9*R*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 37B

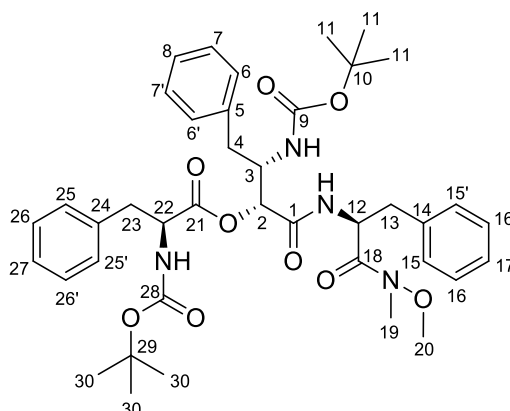


R_f = 0.62 (50% EtOAc/Hexane); ***m.p.*** 98 – 102 °C; **IR (ν_{max}/cm^{-1})**: 3356 (NH), 2973, 2937 (C-H), 1755, 1690, 1656 (C=O), 1526, 1497 (C=C), 1165 (C-O); **1H NMR (300 MHz, $CDCl_3$)** δ 7.45 – 6.99 (18H, m, Ar-*H*), 5.38 – 4.89 and 4.69 – 4.15 (6H, 2xm, 2xNH, *H*-2, *H*-3, *H*-12 and *H*-22), 3.76 – 3.56 (3H, m, *H*-20), 3.29 – 2.83 and 2.75 – 2.45 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.47 – 1.26 (18H, m, *H*-11 and *H*-30); **^{13}C NMR (75 MHz, $CDCl_3$)** δ 171.16, 170.66 and 167.37 (C-21, C-18 and C-1), 155.57 and 154.83 (C-28 and C-9), 137.40 (Ar-C), 136.42 (Ar-C), 135.69 (Ar-C), 129.46 (Ar-C), 129.39 (Ar-C), 129.18 (Ar-C), 128.86 (Ar-C), 128.46 (Ar-C), 128.33 (Ar-C), 127.30 (Ar-C), 126.93 (Ar-C), 126.41 (Ar-C), 80.56 and 79.39 (C-29 and C-10), 74.19 (C-2), 61.56 (C-20), 55.14, 52.91 and 50.60 (C-22, C-12 and C-3), 37.93, 37.81 and 37.52 (C-23, C-13 and C-4), 32.09 (C-19), 28.35 (C-30 and C-11); **HRMS m/z** : calculated for $C_{40}H_{53}N_4O_9$: 733.3813, found $[M + H]^+$: 733.3822.

4.4.18 Synthesis of (2*S*)-(5*S*,9*S*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 38

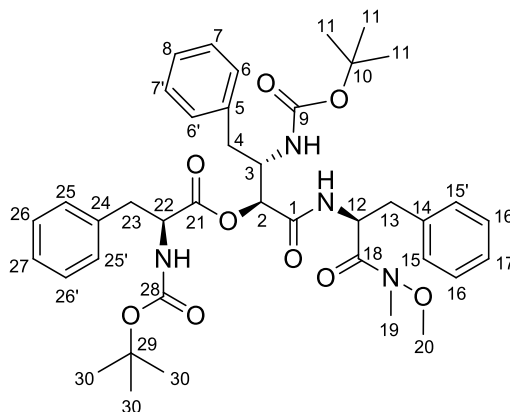
L-phenylalanine isocyanide (125 mg, 0.57 mmol), *N*-Boc-L-phenylalaninal (142 mg, 0.57 mmol) and *N*-Boc-L-phenylalanine (151 mg, 0.57 mmol) were stirred in CH_2Cl_2 (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **38** (0.32 g, 77%) as a (1.78 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **38A** and the minor **38B**.

(2S)-(5S,8R,9S)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 38A



R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 136 – 139 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3358 (NH), 2973, 2938 (C-H), 1755, 1691, 1660 (C=O), 1527, 1497 (C=C), 1165 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.39 – 7.10 (18H, m, Ar-H), 5.32 – 5.15 (2H, m, H-2 and H-12), 5.12 – 5.00 (1H, m, NH), 4.98 – 4.90 (1H, m, NH), 4.48 – 4.35 and 4.29 – 4.13 (2H, 2xm, H-22 and H-3), 3.62 (3H, s, H-20), 3.23 – 2.85 and 2.61 – 2.46 (10H, 2xm, NH, H-4, H-13, H-19 and H-23), 1.47 – 1.27 (18H, m, H-11 and H-30); **^{13}C NMR (75 MHz, CDCl_3)** δ 171.20, 170.81 and 167.43 (C-21, C-18 and C-1), 155.45 and 154.78 (C-28 and C-9), 137.39 (Ar-C), 136.48 (Ar-C), 135.61 (Ar-C), 129.44 (Ar-C), 129.39 (Ar-C), 129.14 (Ar-C), 128.91 (Ar-C), 128.39 (Ar-C), 128.34 (Ar-C), 127.41 (Ar-C), 126.83 (Ar-C), 126.43 (Ar-C), 80.63 and 79.27 (C-29 and C-10), 73.97 (C-2), 61.56 (C-20), 55.22, 52.90 and 50.23 (C-22, C-12 and C-3), 37.99, 37.71 and 37.40 (C-23, C-13 and C-4), 32.08 (C-19), 28.32 (C-30 and C-11); **HRMS m/z** : calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3802.

(2S)-(5S,8S,9S)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 38B

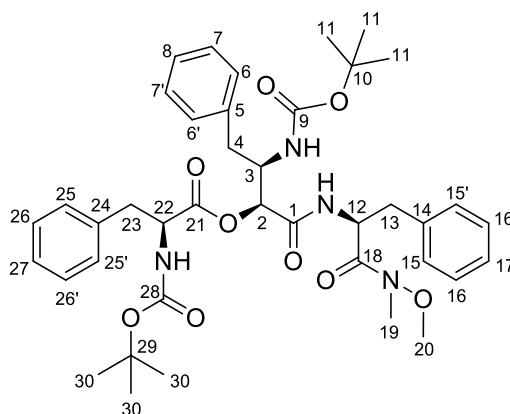


R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 119 – 122 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3357 (NH), 2971, 2939 (C-H), 1756, 1692, 1659 (C=O), 1519, 1497 (C=C), 1164 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.45 – 6.99 (17H, m, Ar-H), 5.38 – 4.86 and 4.70 – 4.13 (6H, 2xm, 2xNH, H-2, H-3, H-12 and H-22), 3.81 – 3.55 (3H, m, H-20), 3.30 – 2.76 and 2.75 – 2.46 (10H, 2xm, NH, H-4, H-13, H-19 and H-23), 1.50 – 1.20 (18H, m, H-11 and H-30); **^{13}C NMR (75 MHz, CDCl_3)** δ 171.15, 170.91 and 166.73 (C-21, C-18 and C-1), 155.37 and 155.15 (C-28 and C-9), 137.37 (Ar-C), 136.09 (Ar-C), 135.95 (Ar-C), 129.47 (Ar-C), 129.36 (Ar-C), 129.16 (Ar-C), 128.79 (Ar-C), 128.44 (Ar-C), 128.26 (Ar-C), 127.23 (Ar-C), 126.93 (Ar-C), 126.40 (Ar-C), 80.47 and 79.45 (C-29 and C-10), 75.05 (C-2), 61.57 (C-20), 55.46, 52.66 and 50.29 (C-22, C-12 and C-3), 38.01, 37.86 and 35.96 (C-23, C-13 and C-4), 32.18 (C-19), 28.32 (C-30 and C-11); **HRMS m/z**: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3802.

4.4.19 Synthesis of (2S)-(5S,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate **39**

L-phenylalanine isocyanide (125 mg, 0.57 mmol), *N*-Boc-D-phenylalaninal (142 mg, 0.57 mmol) and *N*-Boc-L-phenylalanine (151 mg, 0.57 mmol) were stirred in CH_2Cl_2 (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **39** (0.37 g, 89%) as a (1.85 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **39A** and the minor **39B**.

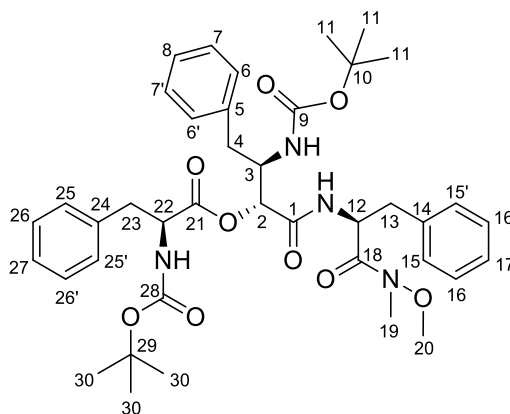
(2S)-(5S,8S,9R)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoate **39A**



R_f = 0.62 (50% EtOAc/Hexane); **m.p.** 68 – 70 °C; **IR** ($\nu_{\max}/\text{cm}^{-1}$): 3320 (NH), 2976, 2936 (C-H), 1753, 1690, 1655 (C=O), 1522, 1497 (C=C), 1163 (C-O); **^1H NMR (300 MHz, CDCl_3)** δ 7.38 – 7.10 (18H, m, Ar-H), 6.97 – 6.83 (1H, m, NH), 5.32 – 5.09 (2H, m, H-2 and H-12), 4.99 – 4.84 (1H, m, NH), 4.66 – 4.53 and 4.43 – 4.24 (2H, 2xm, H-22 and H-3), 3.61 (3H, s, H-20), 3.24 – 2.89 and 2.78 – 2.41 (10H, 2xm, NH, H-

4, *H*-13, *H*-19 and *H*-23), 1.51 – 1.21 (18H, m, *H*-11 and *H*-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.09, 170.89 and 167.37 (C-21, C-18 and C-1), 155.18 and 154.38 (C-28 and C-9), 137.34 (Ar-C), 136.04 (Ar-C), 135.97 (Ar-C), 129.47 (Ar-C), 129.37 (Ar-C), 129.17 (Ar-C), 128.88 (Ar-C), 128.41 (Ar-C), 128.27 (Ar-C), 127.24 (Ar-C), 126.95 (Ar-C), 126.41 (Ar-C), 80.50 and 79.40 (C-29 and C-10), 75.09 (C-2), 61.51 (C-20), 54.73, 52.56 and 50.25 (C-22, C-12 and C-3), 37.86, 37.72 and 37.35 (C-23, C-13 and C-4), 32.13 (C-19), 28.33 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3799.

(2*S*)-(5*S*,8*R*,9*R*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 39B

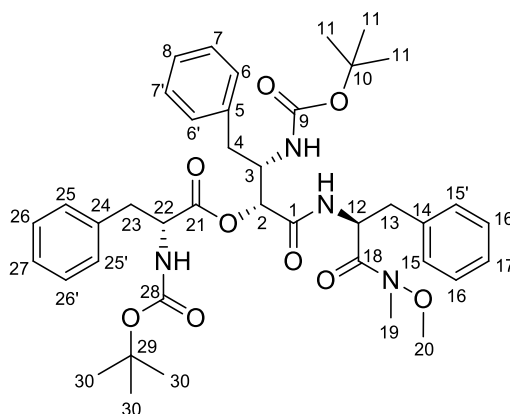


R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 71 – 74 °C; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3311 (NH), 2974, 2938 (C-H), 1753, 1689, 1655 (C=O), 1497 (C=C), 1160 (C-O); ^1H NMR (300 MHz, CDCl_3) δ 7.48 – 7.08 (17H, m, Ar-*H*), 5.46 – 4.83 and 4.76 – 4.28 (6H, 2xm, 2xNH, *H*-2, *H*-3, *H*-12 and *H*-22), 3.75 – 3.66 (3H, m, *H*-20), 3.22 – 2.87 and 2.79 – 2.58 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.55 – 1.21 (18H, m, *H*-11 and *H*-30); ^{13}C NMR (75 MHz, CDCl_3) δ 171.09, 170.94 and 167.39 (C-21, C-18 and C-1), 155.18 and 154.39 (C-28 and C-9), 137.36 (Ar-C), 136.66 (Ar-C), 136.07 (Ar-C), 129.47 (Ar-C), 129.37 (Ar-C), 129.17 (Ar-C), 128.88 (Ar-C), 128.46 (Ar-C), 128.27 (Ar-C), 127.26 (Ar-C), 126.95 (Ar-C), 126.41 (Ar-C), 80.47 and 79.44 (C-29 and C-10), 75.03 (C-2), 61.57 (C-20), 54.67, 52.61 and 50.28 (C-22, C-12 and C-3), 38.04, 37.85 and 37.38 (C-23, C-13 and C-4), 32.13 (C-19), 28.33 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3799.

4.4.20 Synthesis of (2*R*)-(5*S*,9*S*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxa-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 40

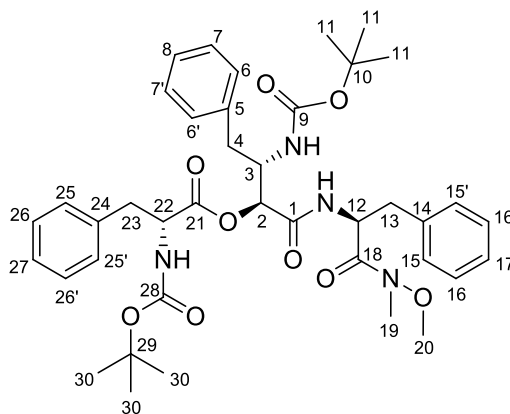
L-phenylalanine isocyanide (125 mg, 0.57 mmol), *N*-Boc-L-phenylalaninal (142 mg, 0.57 mmol) and *N*-Boc-D-phenylalanine (151 mg, 0.57 mmol) were stirred in CH₂Cl₂ (4 ml) at room temperature for 5 d. The preparation was completed using the same procedure as described in 4.4.9 to yield **40** (0.41 g, 98%) as a (2.03 : 1.00) mixture of diastereomers. The mixture was then separated by preparative HPLC to obtain two pure diastereomers, the major being **40A** and the minor **40B**.

(2*R*)-(5*S*,8*R*,9*S*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 40A



R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 65 – 67 °C; IR ($\nu_{\text{max}}/\text{cm}^{-1}$): 3318 (NH), 2974, 2938 (C-H), 1746, 1690, 1654 (C=O), 1497 (C=C), 1160 (C-O); ¹H NMR (300 MHz, CDCl₃) δ 7.43 – 6.96 (17H, m, Ar-H), 5.31 – 5.05 (2H, m, *H*-2 and *H*-12), 4.98 – 4.72 (2H, m, 2xNH), 4.56 – 4.40 and 4.40 – 4.21 (2H, 2xm, *H*-22 and *H*-3), 3.66 (3H, s, *H*-20), 3.21 – 2.83 and 2.74 – 2.60 (10H, 2xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.53 – 1.19 (18H, m, *H*-11 and *H*-30); ¹³C NMR (75 MHz, CDCl₃) δ 171.07, 170.41 and 167.40 (C-21, C-18 and C-1), 155.59 and 154.77 (C-28 and C-9), 137.33 (Ar-C), 136.38 (Ar-C), 135.95 (Ar-C), 129.45 (Ar-C), 129.34 (Ar-C), 129.18 (Ar-C), 128.86 (Ar-C), 128.44 (Ar-C), 128.39 (Ar-C), 127.24 (Ar-C), 126.87 (Ar-C), 126.51 (Ar-C), 80.63 and 79.42 (C-29 and C-10), 74.46 (C-2), 61.52 (C-20), 54.66, 52.87 and 50.67 (C-22, C-12 and C-3), 37.85, 37.66 and 37.39 (C-23, C-13 and C-4), 32.17 (C-19), 28.35 (C-30 and C-11); HRMS *m/z*: calculated for C₄₀H₅₃N₄O₉: 733.3813, found [M + H]⁺: 733.3815.

(2*R*)-(5*S*,8*S*,9*S*)-5,9-dibenzyl-3,13,13-trimethyl-4,7,11-trioxo-2,12-dioxo-3,6,10-triazatetradecan-8-yl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate 40B



R_f = 0.62 (50% EtOAc/Hexane); *m.p.* 70 – 71 °C; IR ($\nu_{\max}/\text{cm}^{-1}$): 3317 (NH), 2973, 2940 (C-H), 1757, 1689, 1656 (C=O), 1497 (C=C), 1164, 1055 (C-O); $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.45 – 7.02 (18H, m, Ar-*H*), 5.49 – 4.77 and 4.61 – 4.11 (6H, 2xm, 2xNH, *H*-2, *H*-3, *H*-12 and *H*-22), 3.71 – 3.58 (3H, m, *H*-20), 3.23 – 2.84, 2.82 – 2.60 and 2.59 – 2.40 (10H, 3xm, NH, *H*-4, *H*-13, *H*-19 and *H*-23), 1.54 – 1.20 (18H, m, *H*-11 and *H*-30); $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 171.15, 170.91 and 166.73 (C-21, C-18 and C-1), 155.37 and 155.15 (C-28 and C-9), 137.37 (Ar-C), 136.09 (Ar-C), 135.95 (Ar-C), 129.47 (Ar-C), 129.36 (Ar-C), 129.16 (Ar-C), 128.79 (Ar-C), 128.44 (Ar-C), 128.26 (Ar-C), 127.23 (Ar-C), 126.93 (Ar-C), 126.40 (Ar-C), 80.47 and 79.45 (C-29 and C-10), 75.05 (C-2), 61.57 (C-20), 55.46, 52.66 and 50.29 (C-22, C-12 and C-3), 38.01, 37.86 and 35.96 (C-23, C-13 and C-4), 32.18 (C-19), 28.32 (C-30 and C-11); HRMS *m/z*: calculated for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_9$: 733.3813, found $[\text{M} + \text{H}]^+$: 733.3815.

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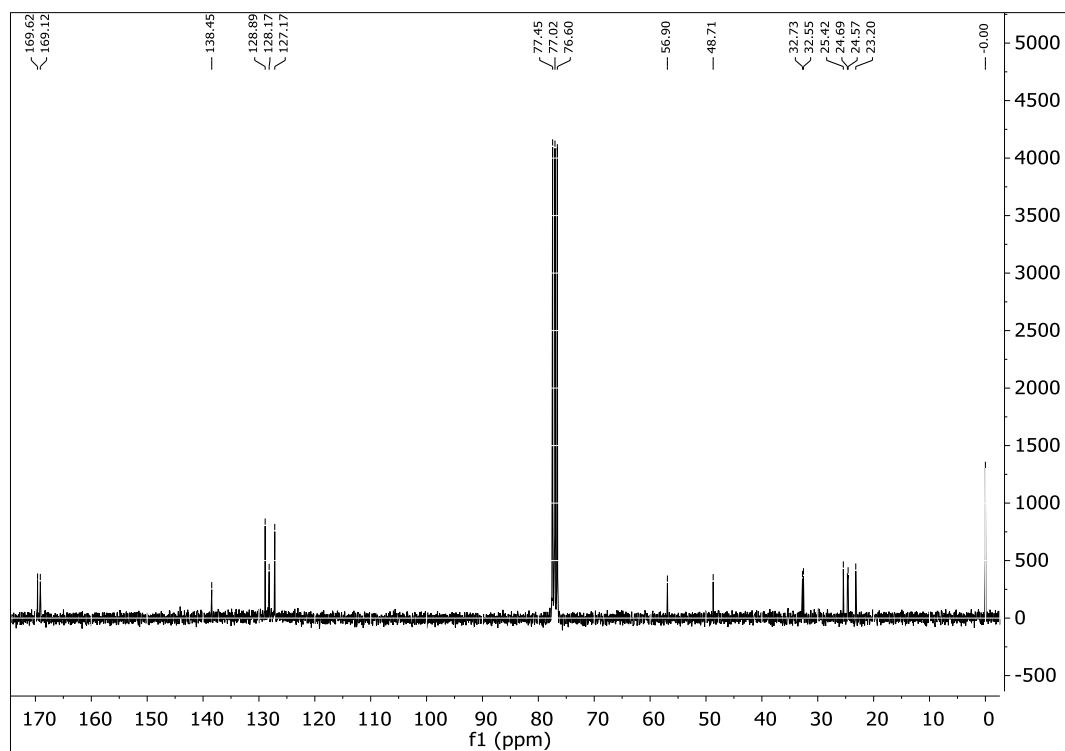
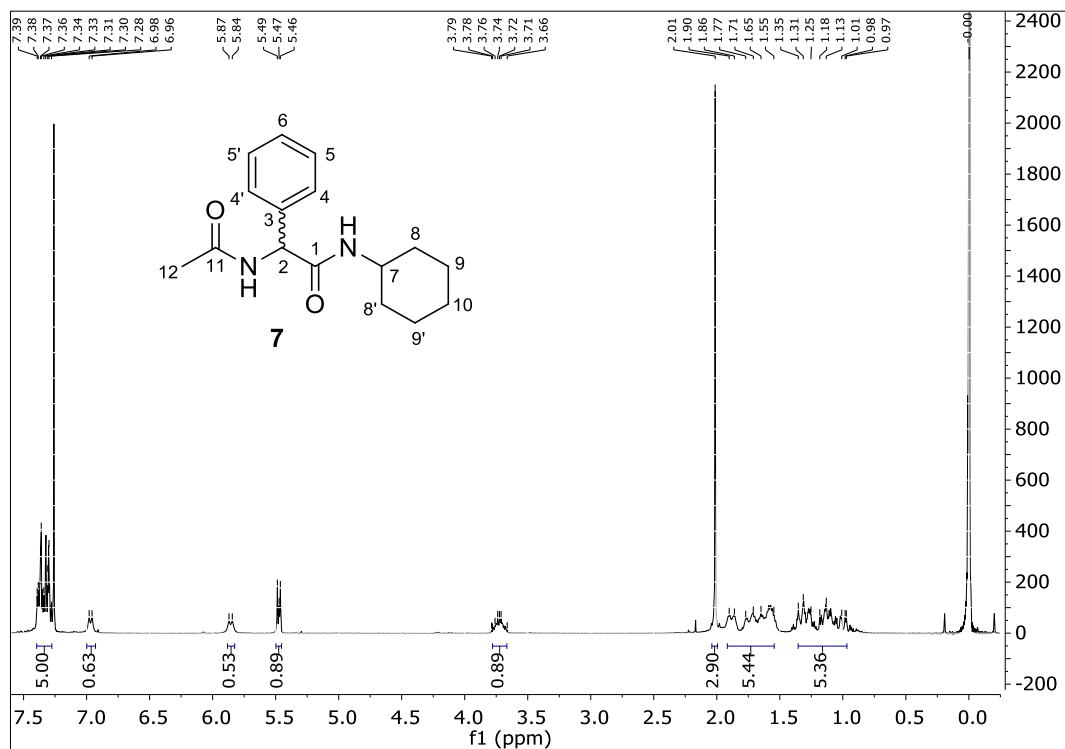
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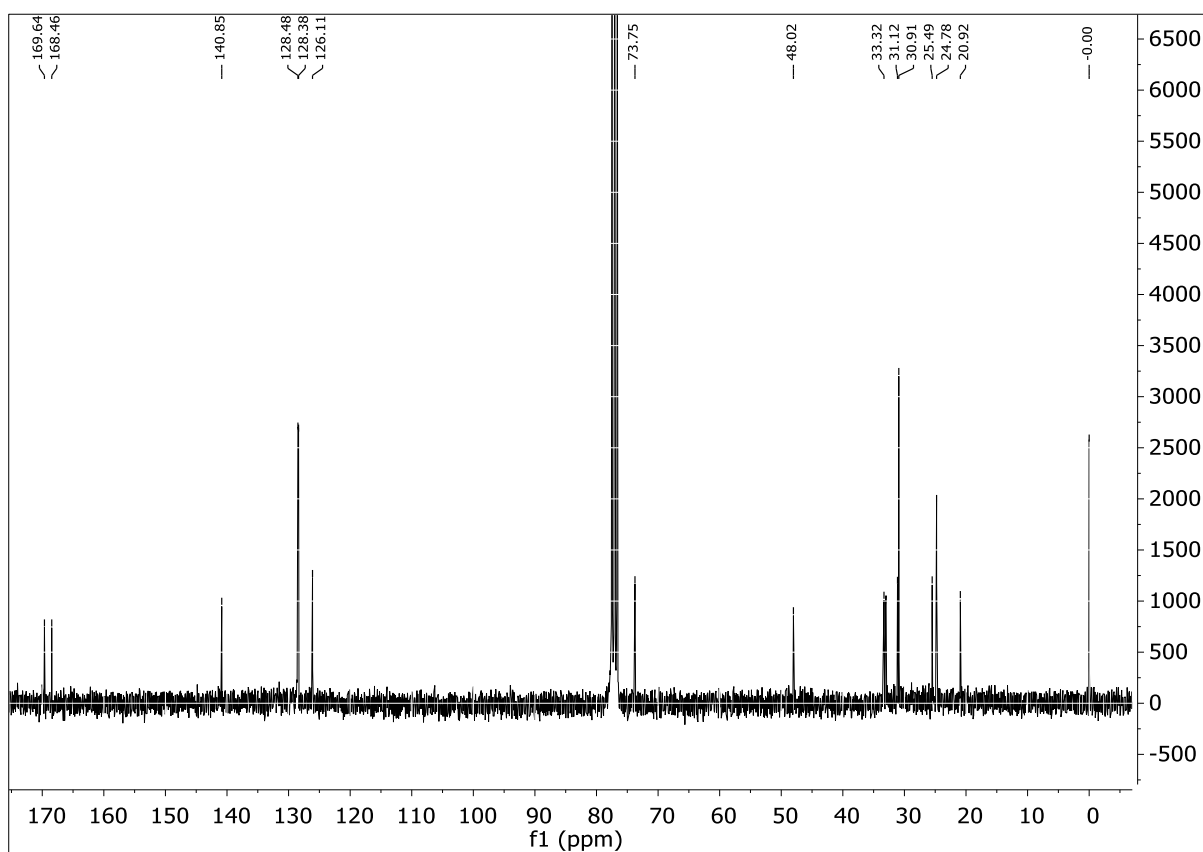
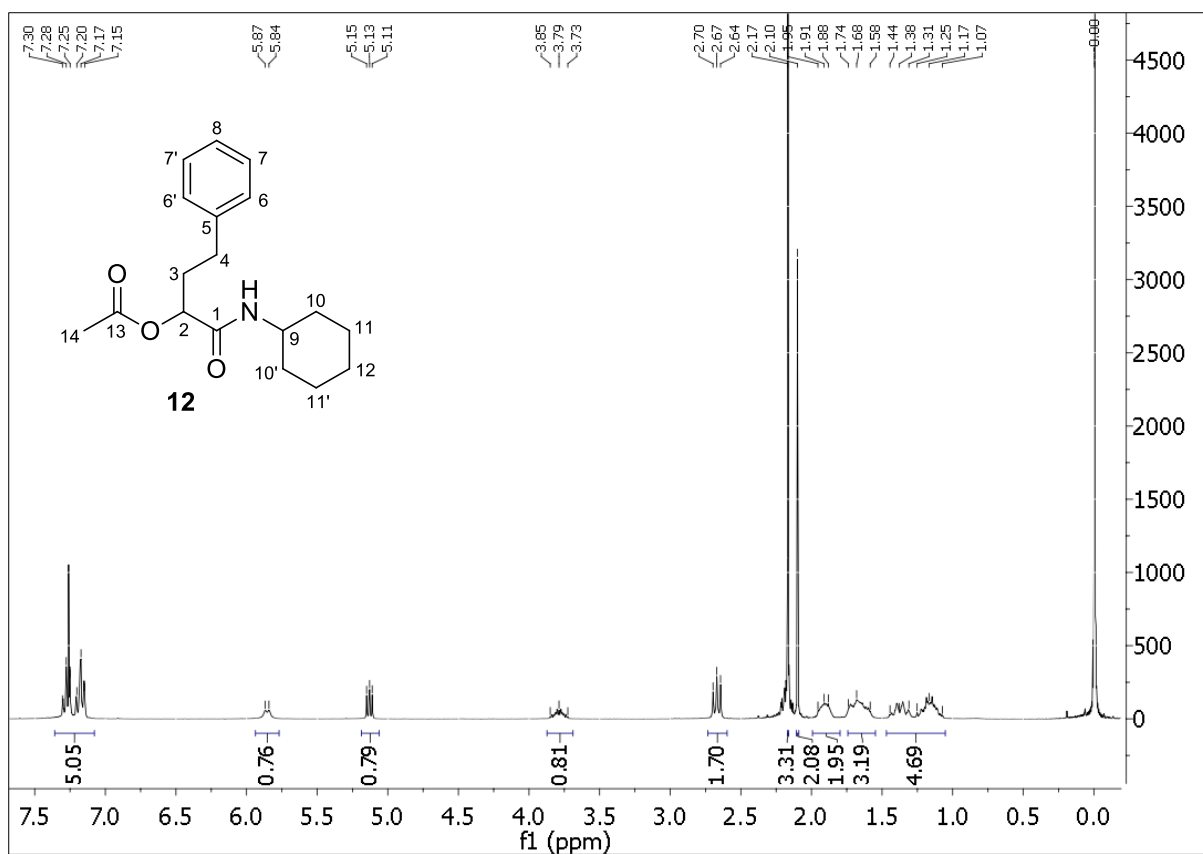
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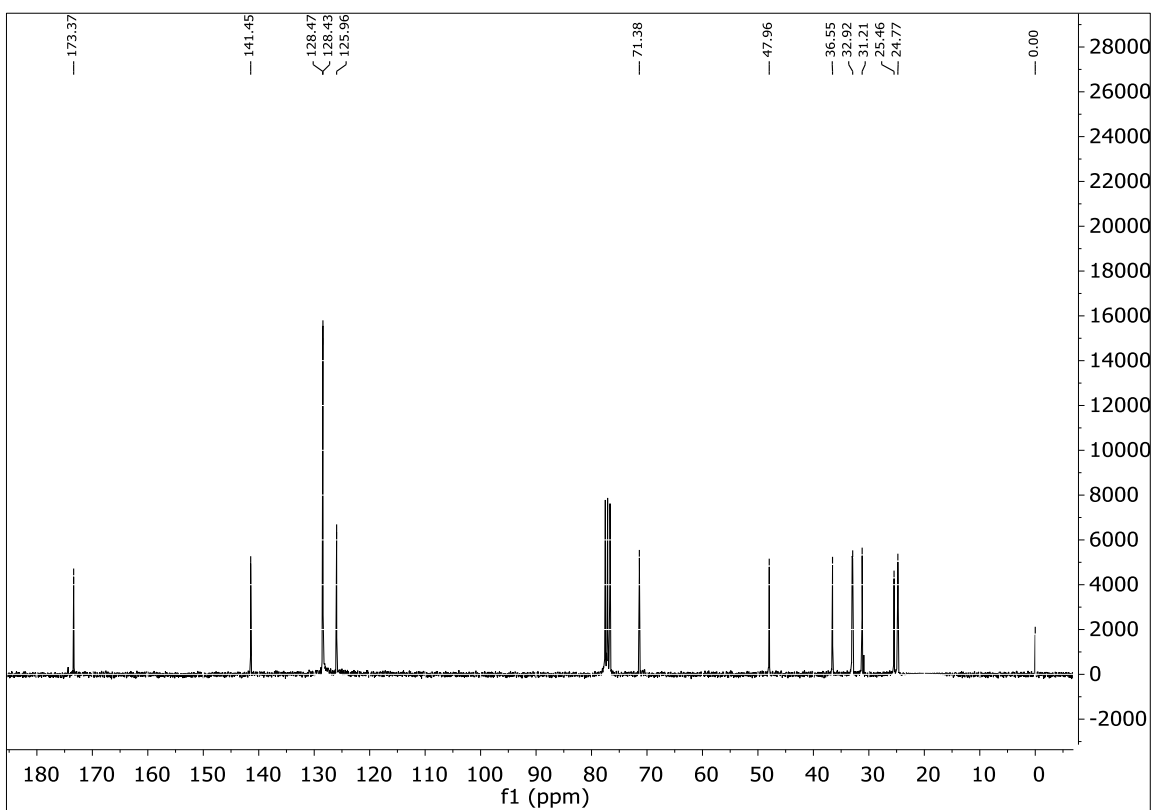
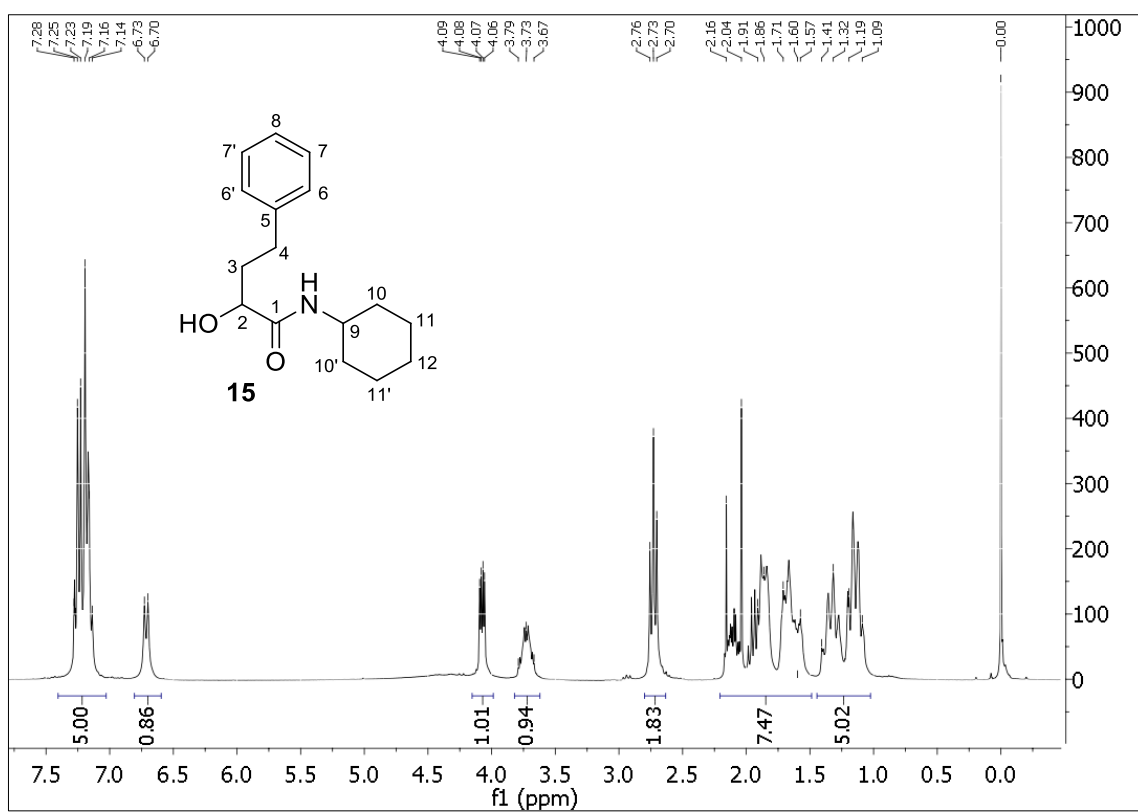
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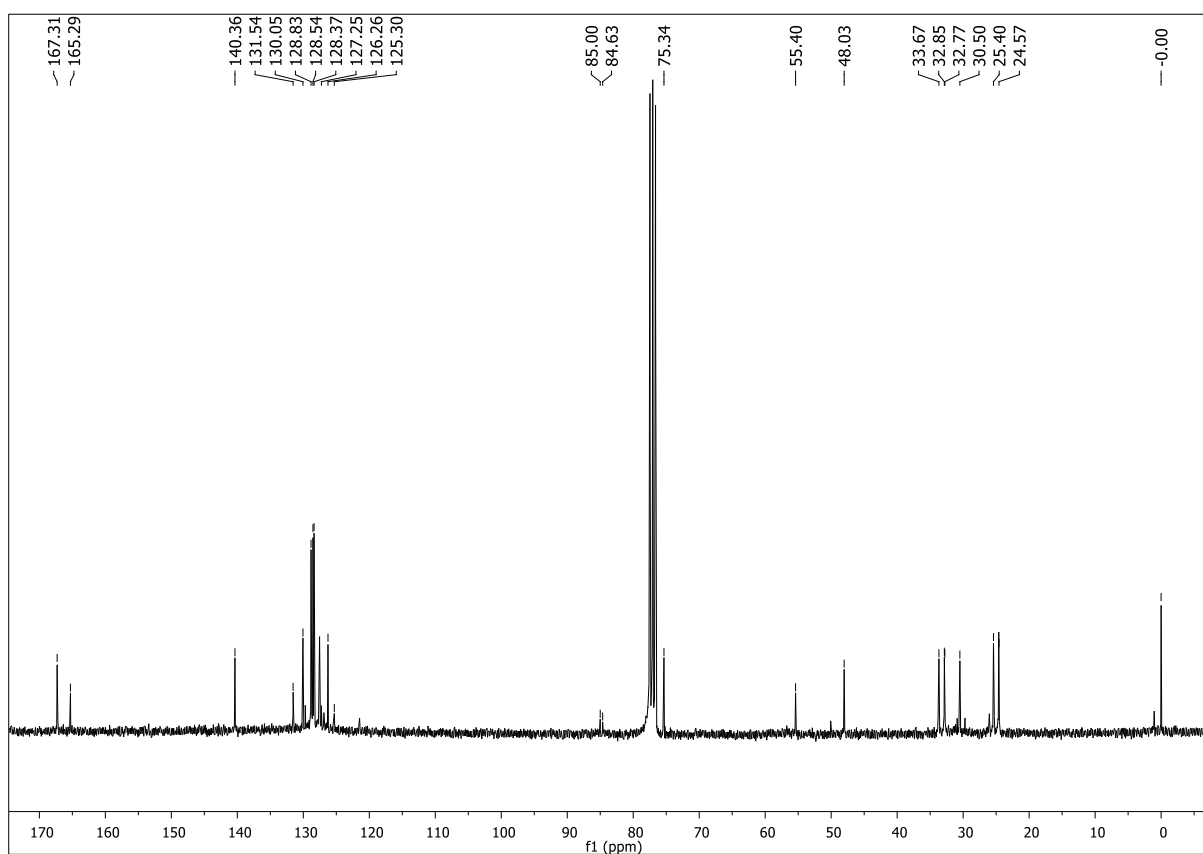
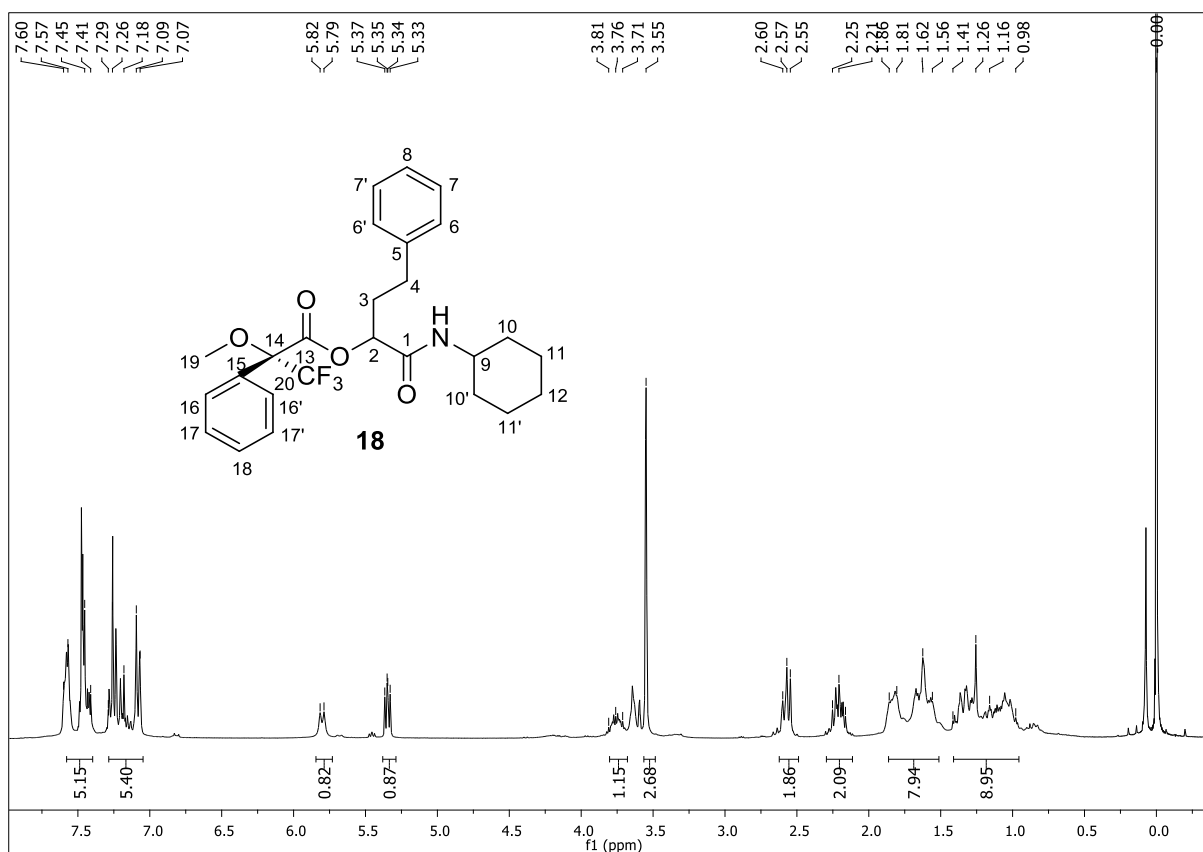
Appendix

A1: Selected ^1H , ^{13}C and ^{19}F NMR Spectra

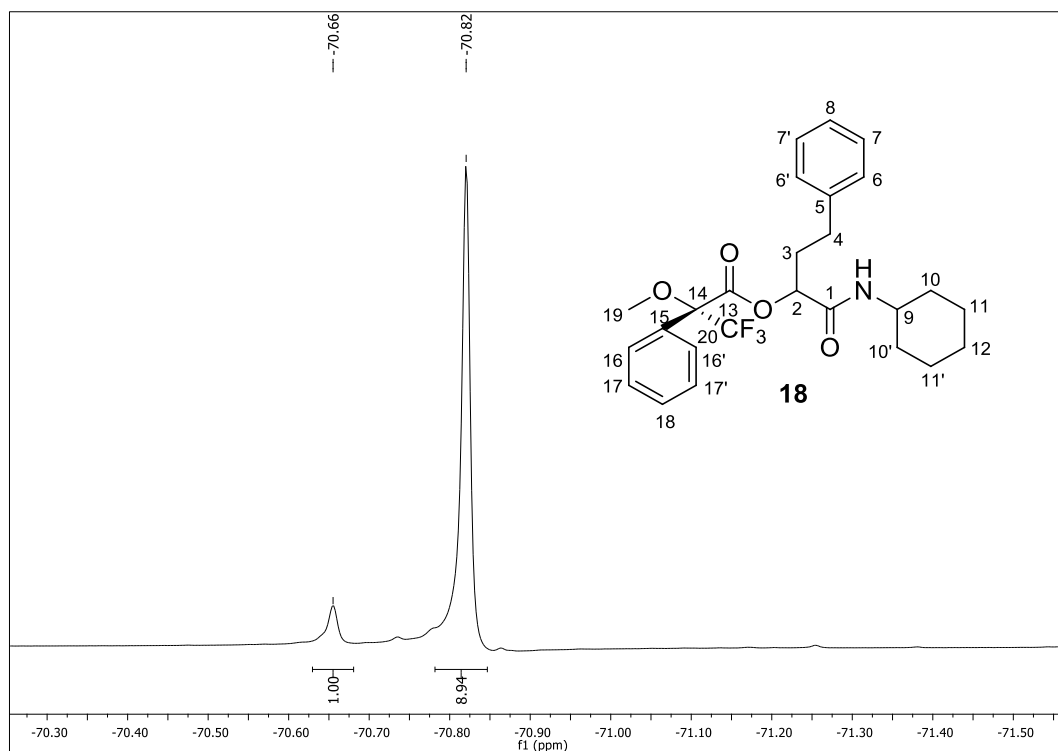




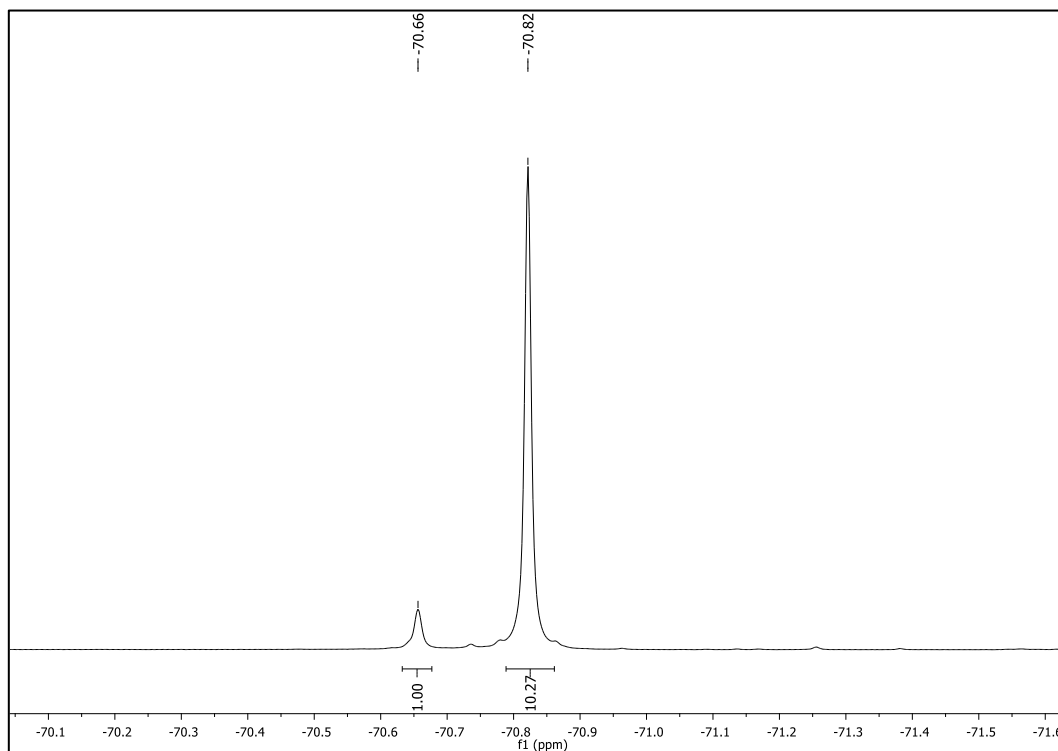


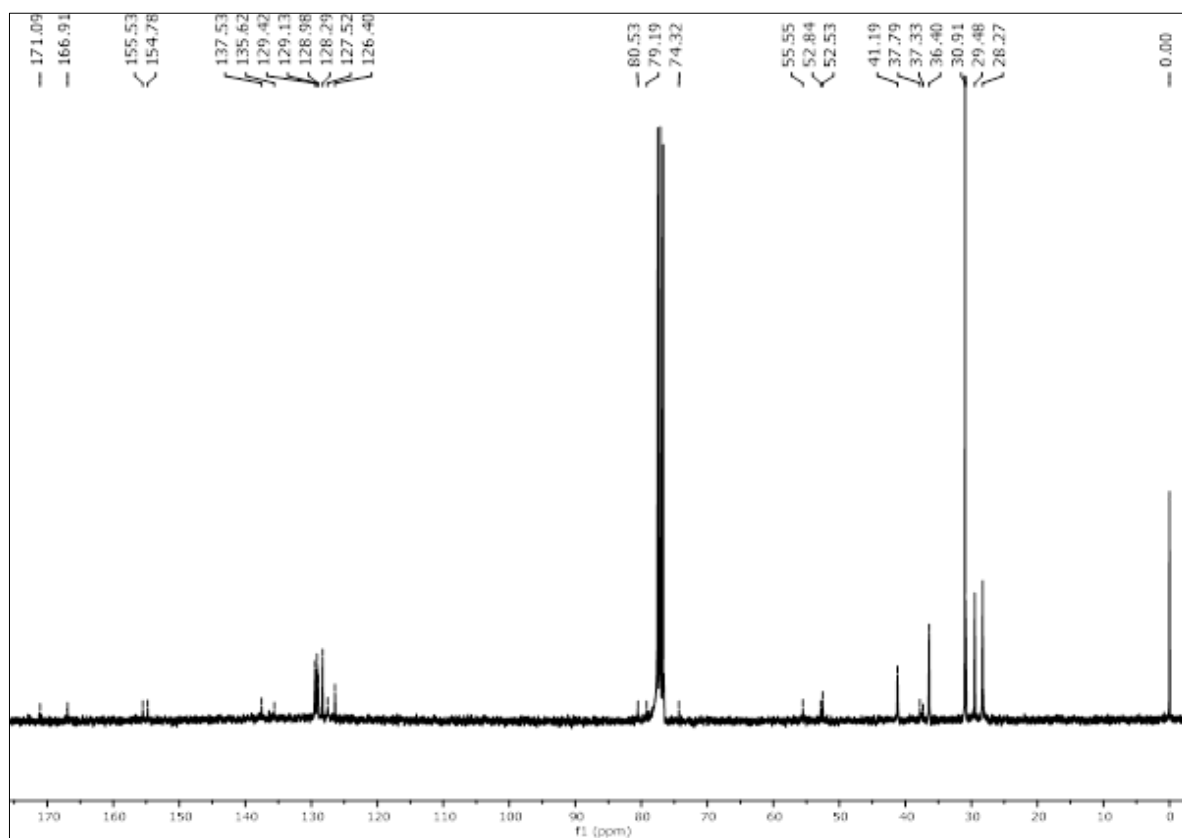
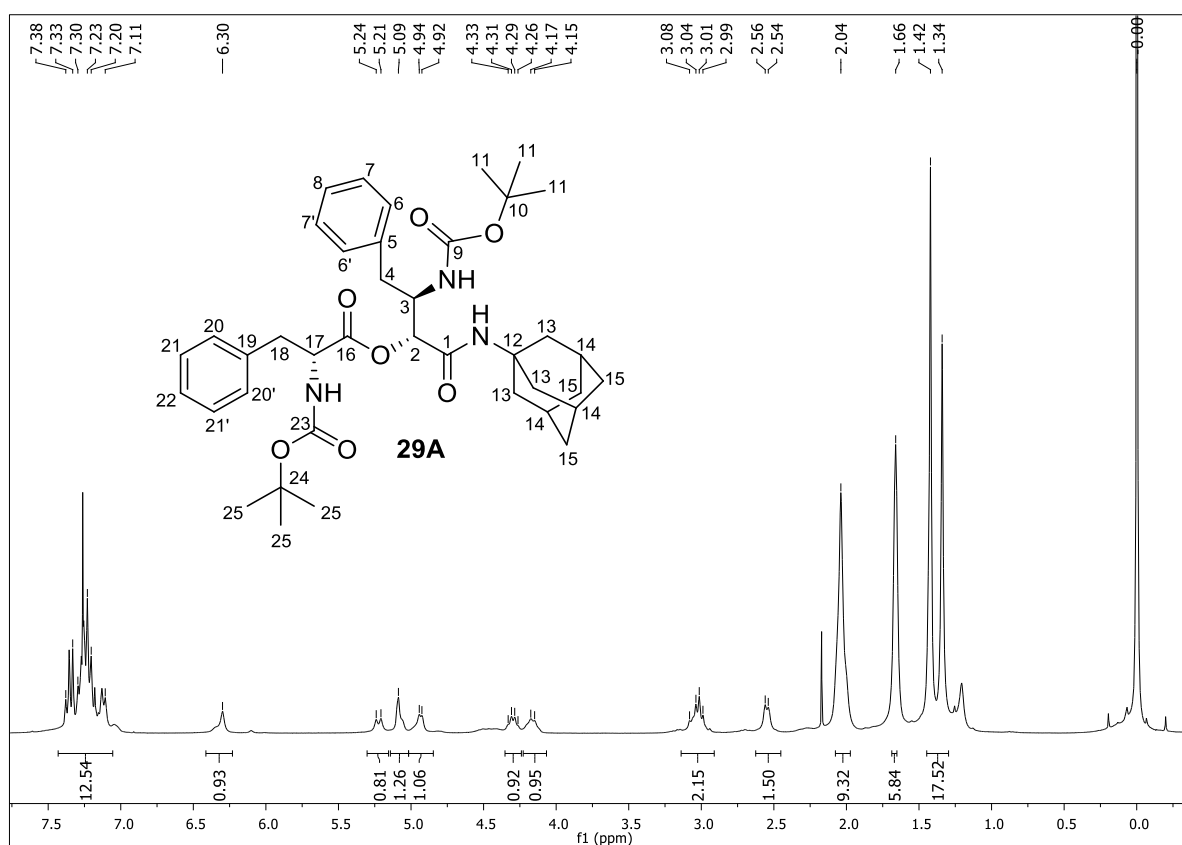


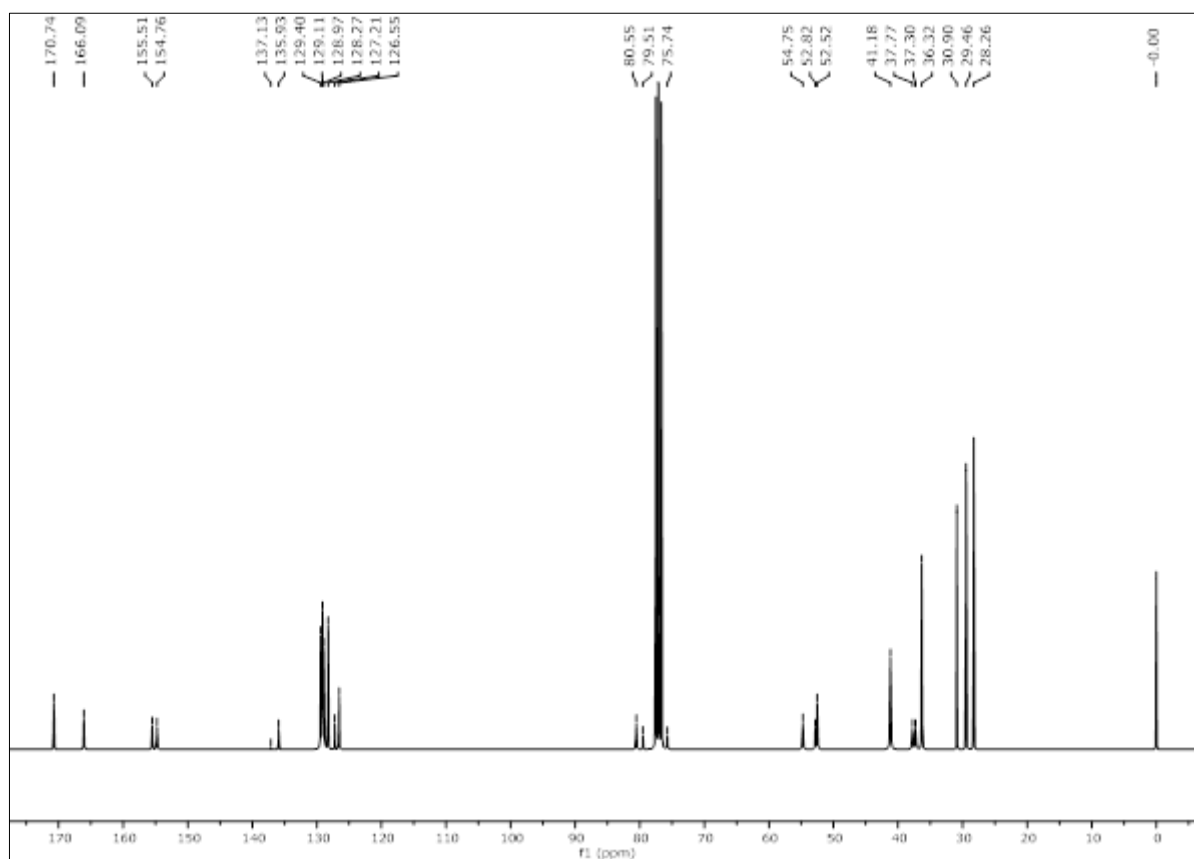
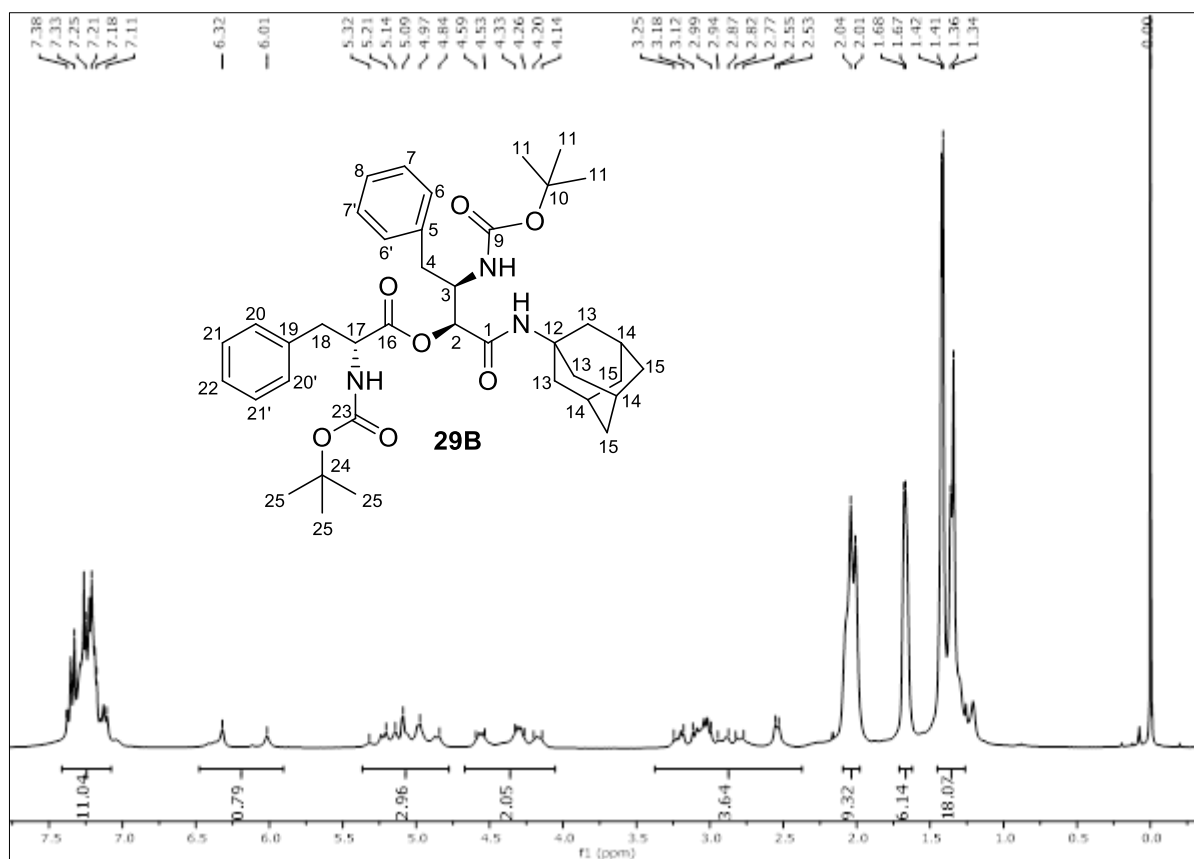
^{19}F NMR Spectrum of 18 from Lipase 6Y reaction

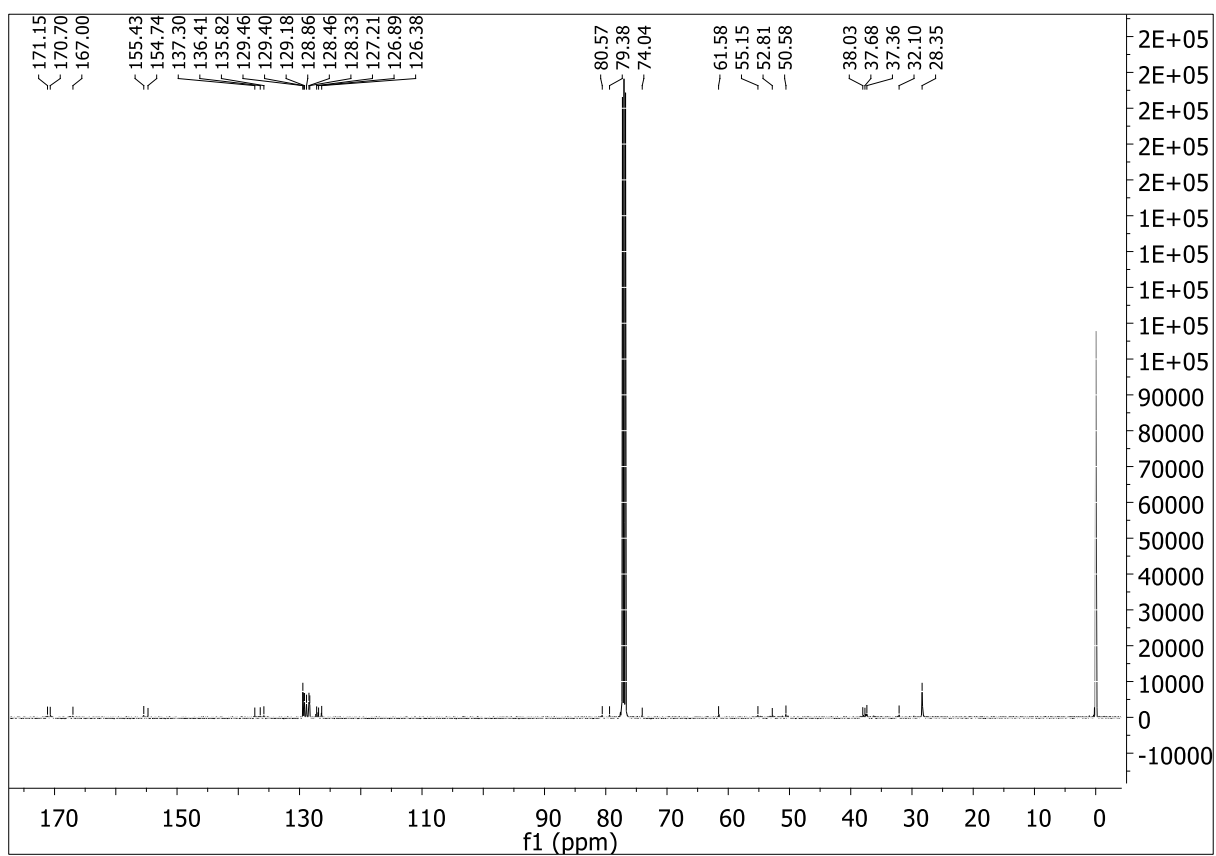
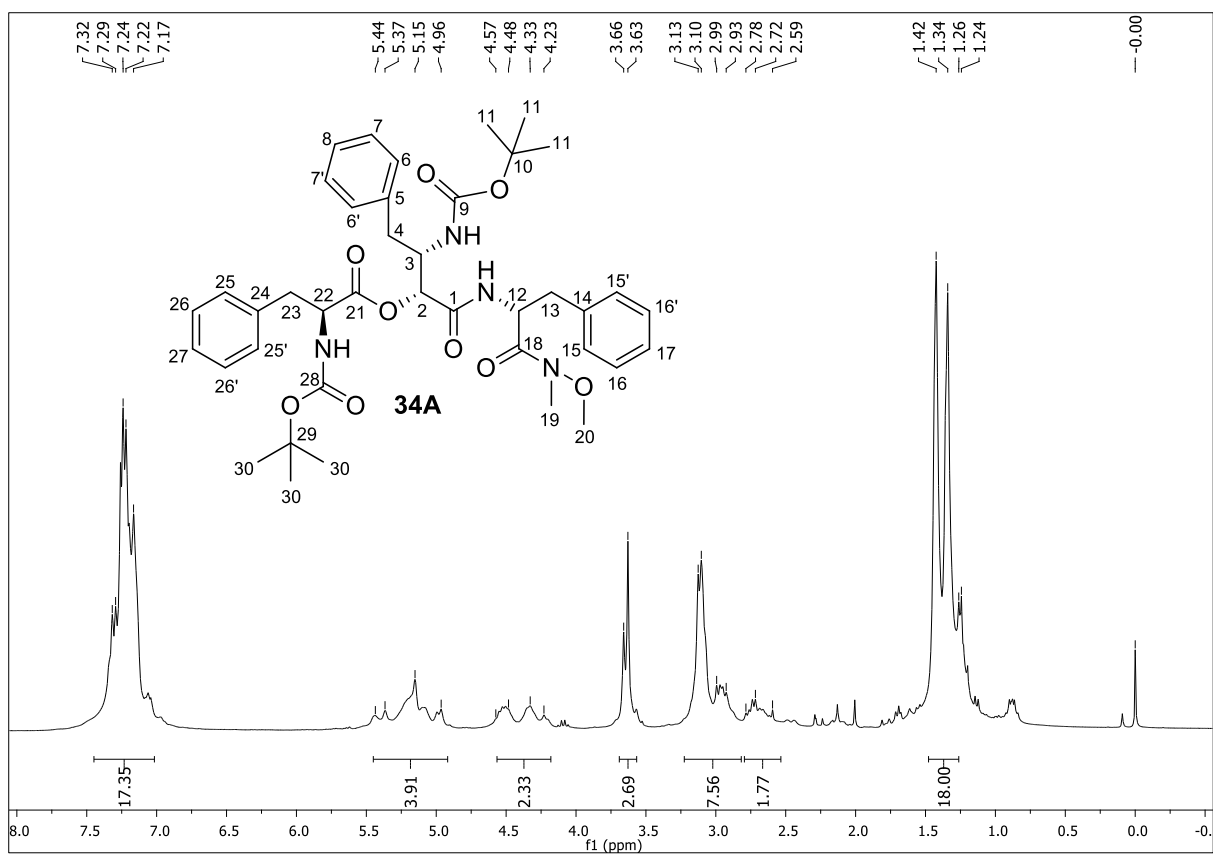


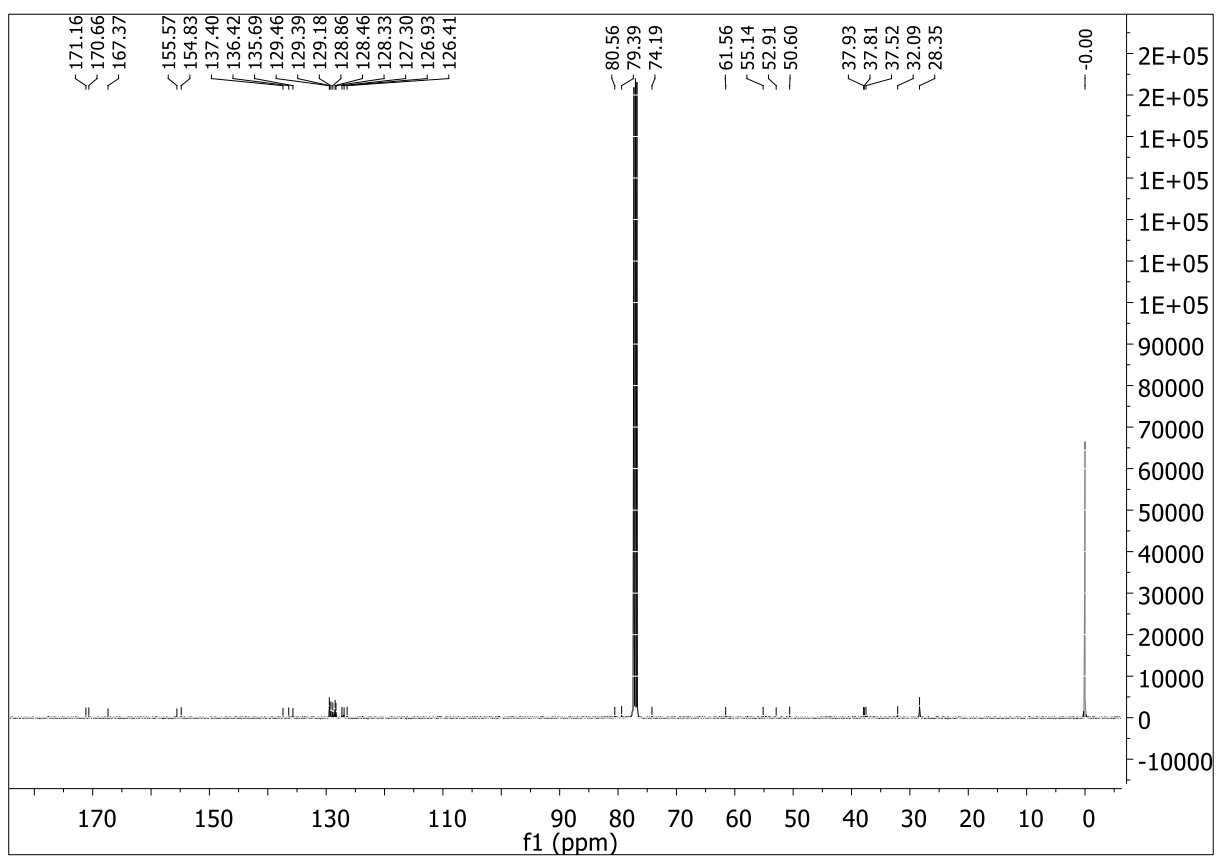
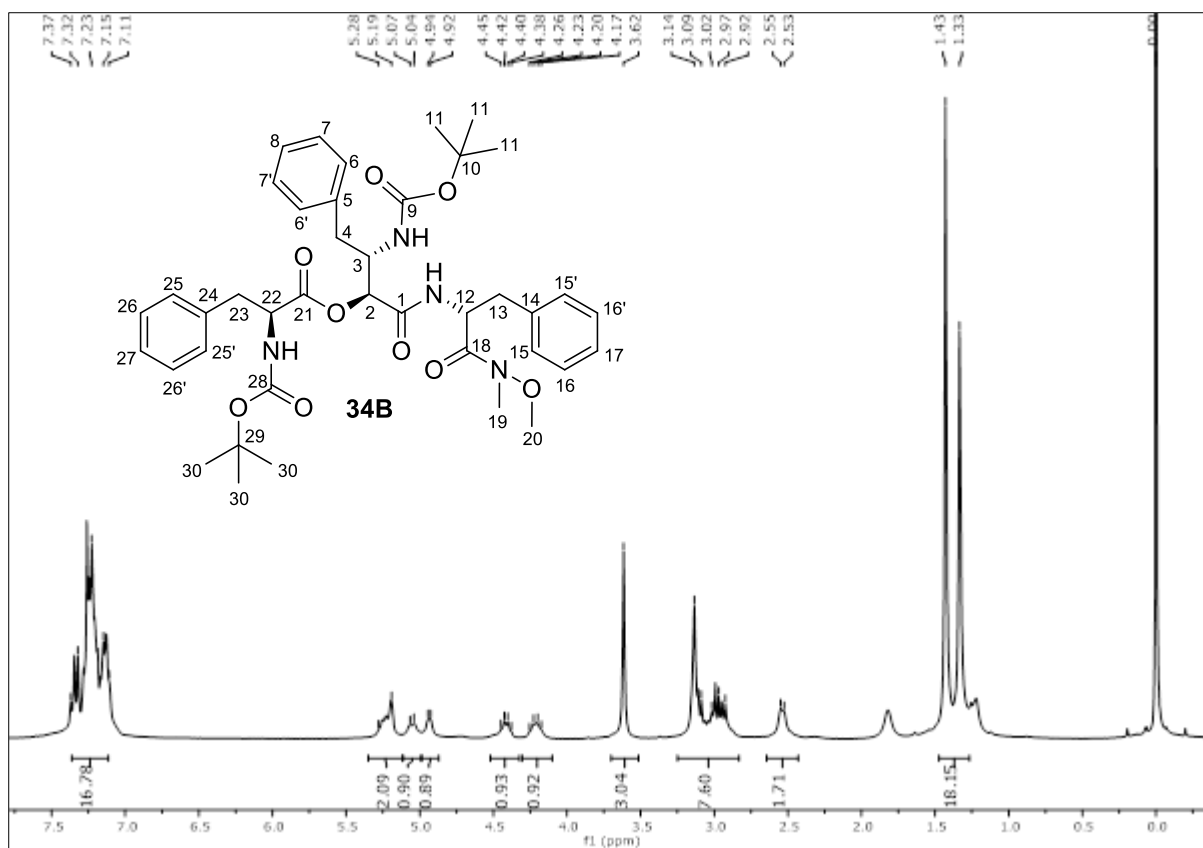
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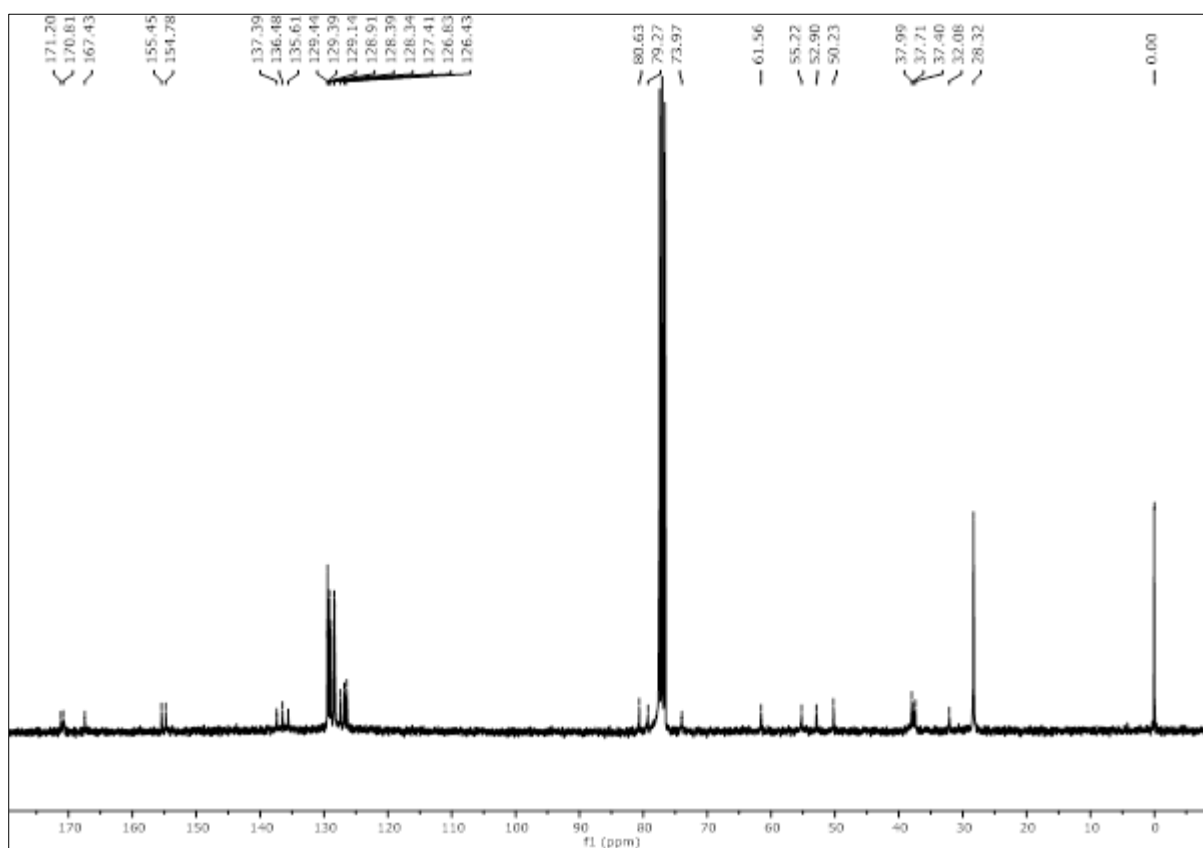
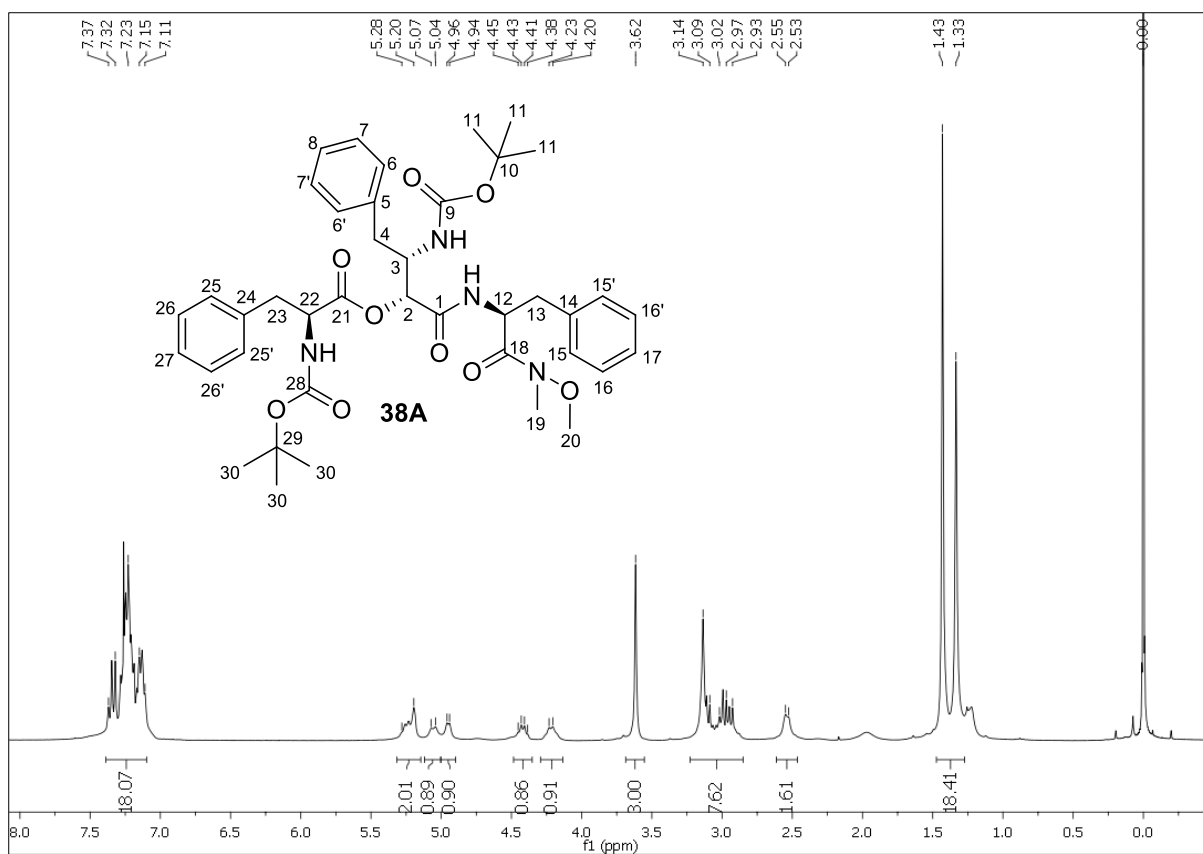


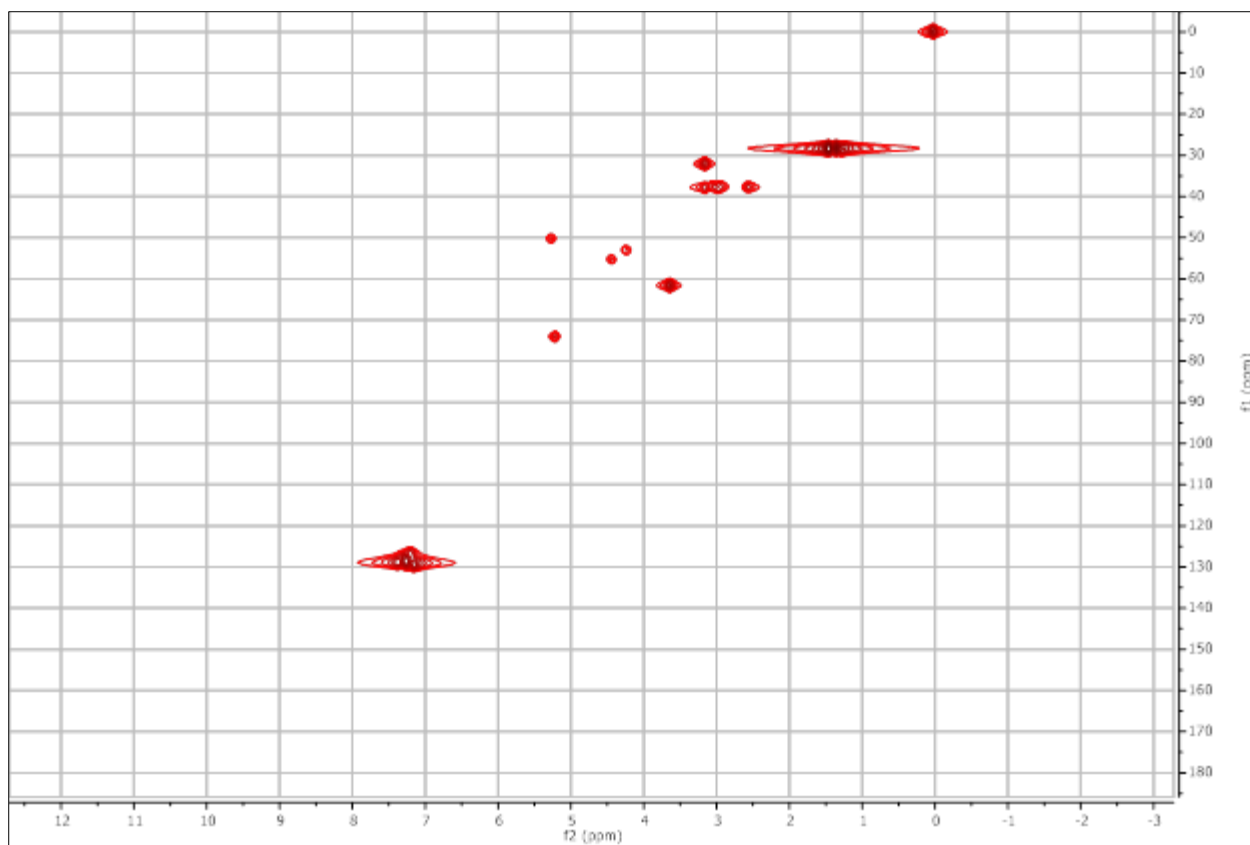
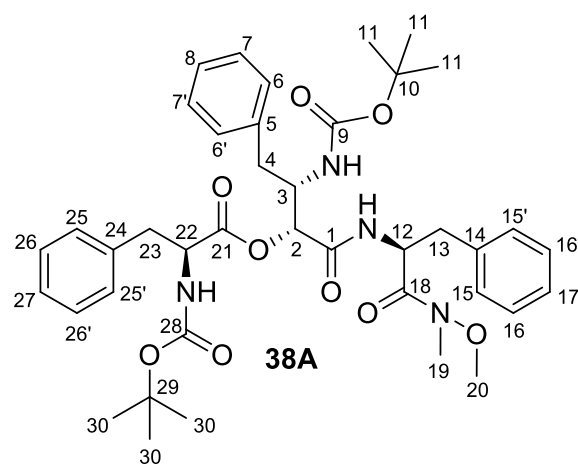


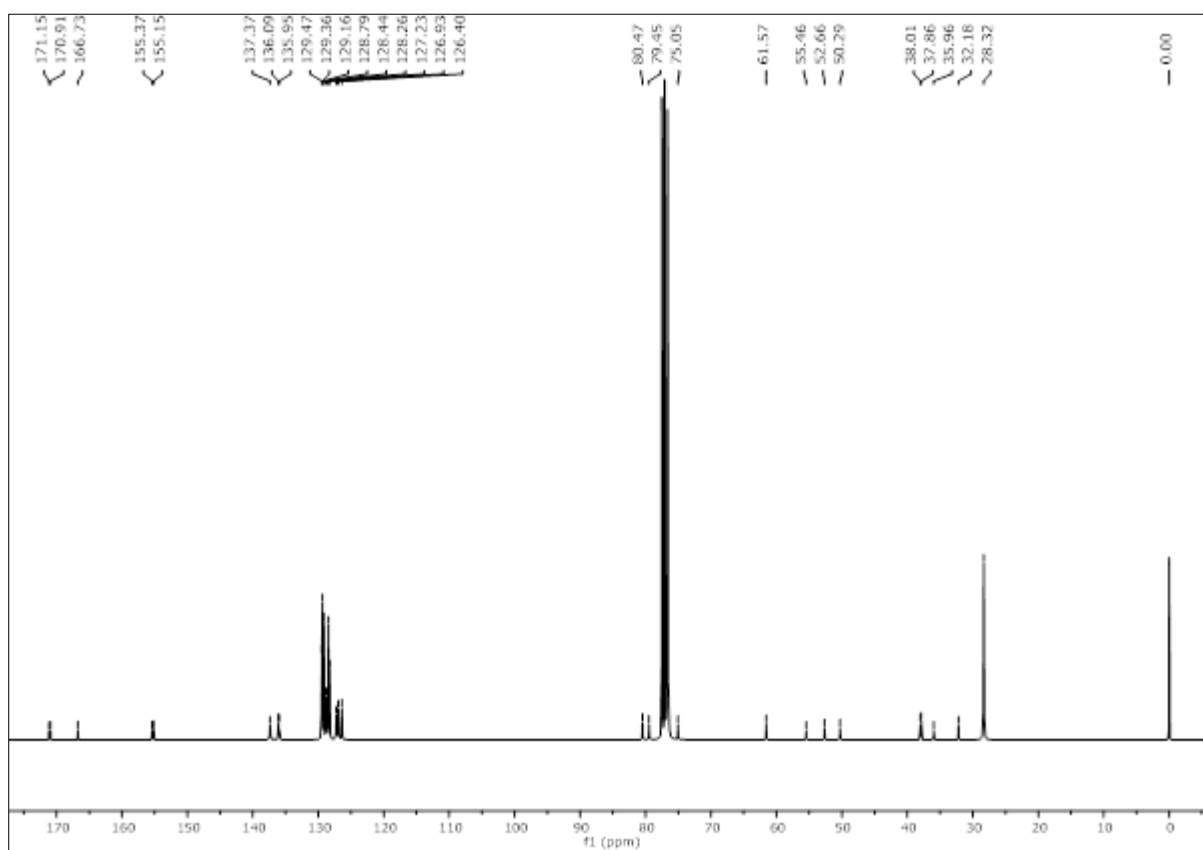
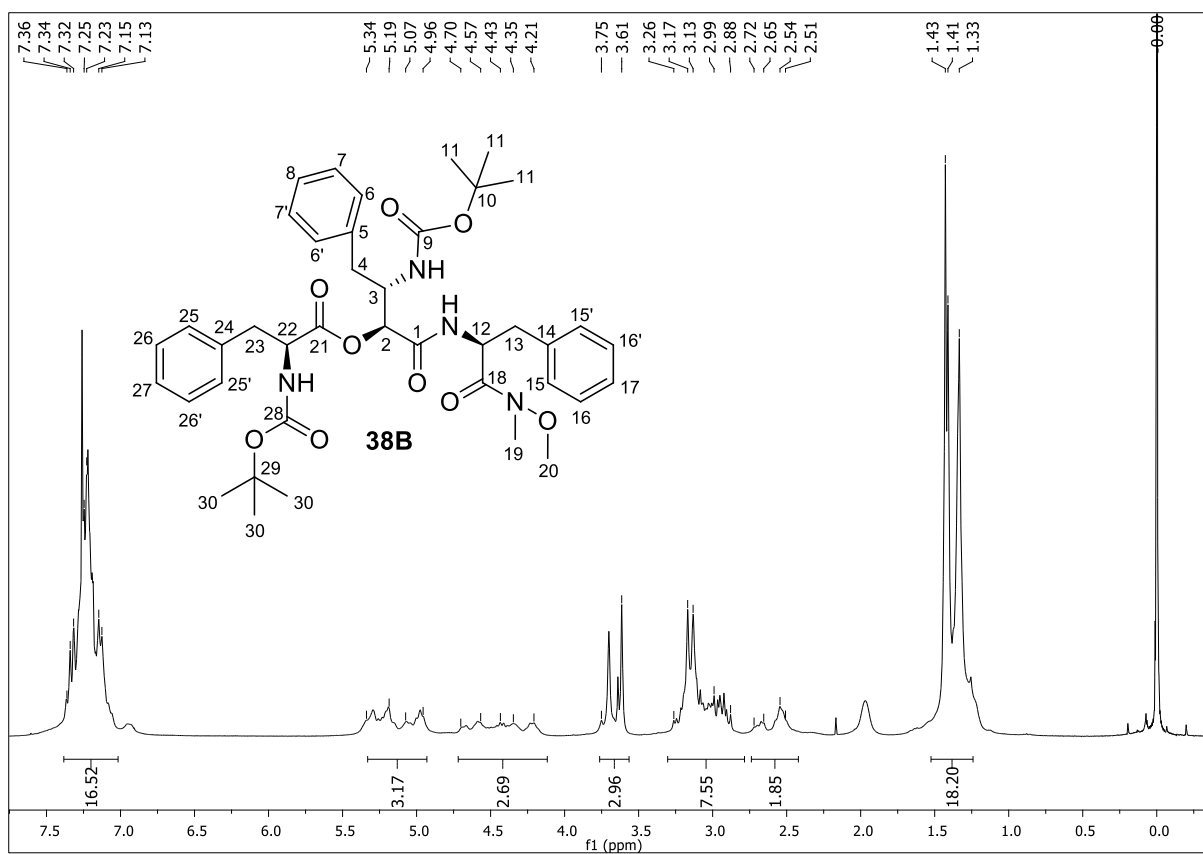












A2: Example of an HPLC method used for separation of Passerini compounds (Gradient elution)

```

Pressure.LowerLimit = 0 [bar]
    Pressure.UpperLimit = 350 [bar]
    MaximumFlowRampDown = 6.000 [ml/min2]
    MaximumFlowRampUp = 6.000 [ml/min2]
    %A.Equate = "%A"
    %B.Equate = "Acetonitrile"
    %C.Equate = "Water"
    %D.Equate = "%D"
    Pump_Pressure.Step = Auto
    Pump_Pressure.Average = On
    Data_Collection_Rate = 10.00 [Hz]
    Rise_Time = 0.20 [s]
    UV_VIS_1.Wavelength = 225 [nm]
    UV_VIS_1.Bandwidth = 4 [nm]
    UV_VIS_1.RefWavelength = Off
    UV_VIS_1.RefBandwidth = 1 [nm]
    UV_VIS_2.Wavelength = 275 [nm]
    UV_VIS_2.Bandwidth = 4 [nm]
    UV_VIS_2.RefWavelength = Off
    UV_VIS_2.RefBandwidth = 1 [nm]
    UV_VIS_3.Wavelength = 330 [nm]
    UV_VIS_3.Bandwidth = 4 [nm]
    UV_VIS_3.RefWavelength = Off
    UV_VIS_3.RefBandwidth = 1 [nm]
    UV_VIS_4.Wavelength = 400 [nm]
    UV_VIS_4.Bandwidth = 4 [nm]
    UV_VIS_4.RefWavelength = Off
    UV_VIS_4.RefBandwidth = 1 [nm]
    UV_VIS_5.Wavelength = 550 [nm]
    UV_VIS_5.Bandwidth = 4 [nm]
    UV_VIS_5.RefWavelength = Off
    UV_VIS_5.RefBandwidth = 1 [nm]
    3DFIELD.RefWavelength = 380 [nm]
    3DFIELD.RefBandwidth = 50 [nm]
    3DFIELD.MinWavelength = 300 [nm]
    3DFIELD.MaxWavelength = 700 [nm]
    3DFIELD.BunchWidth = 3 [nm]

0.000    Autozero
        Wait
        Flow = 1.000 [ml/min]
        %B = 60.0 [%]
        %C = 40.0 [%]
        %D = 0.0 [%]
        Inject
        Pump_Pressure.AcqOn
        UV_VIS_1.AcqOn
        UV_VIS_2.AcqOn
        UV_VIS_3.AcqOn
        UV_VIS_4.AcqOn
        UV_VIS_5.AcqOn

```

```

3DFIELD.AcqOn
Flow = 1.000 [ml/min]
%B = 60.0 [%]
%C = 40.0 [%]
%D = 0.0 [%]

10.000 Flow = 1.000 [ml/min]
      %B = 60.0 [%]
      %C = 40.0 [%]
      %D = 0.0 [%]

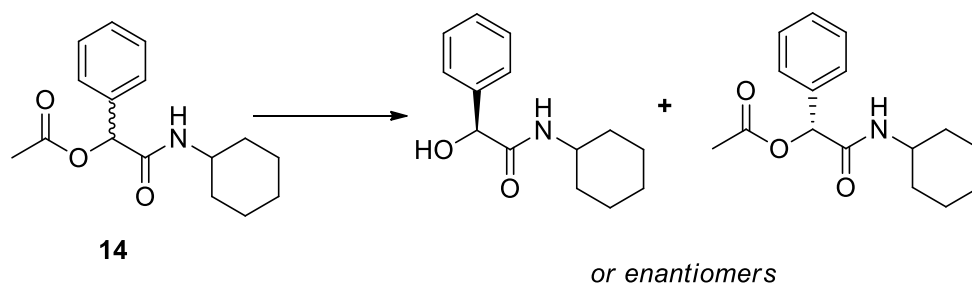
12.000 Flow = 1.000 [ml/min]
      %B = 90.0 [%]
      %C = 10.0 [%]
      %D = 0.0 [%]

35.000 Pump_Pressure.AcqOff
      UV_VIS_1.AcqOff
      UV_VIS_2.AcqOff
      UV_VIS_3.AcqOff
      UV_VIS_4.AcqOff
      UV_VIS_5.AcqOff
      3DFIELD.AcqOff
      Flow = 1.000 [ml/min]
      %B = 90.0 [%]
      %C = 10.0 [%]
      %D = 0.0 [%]

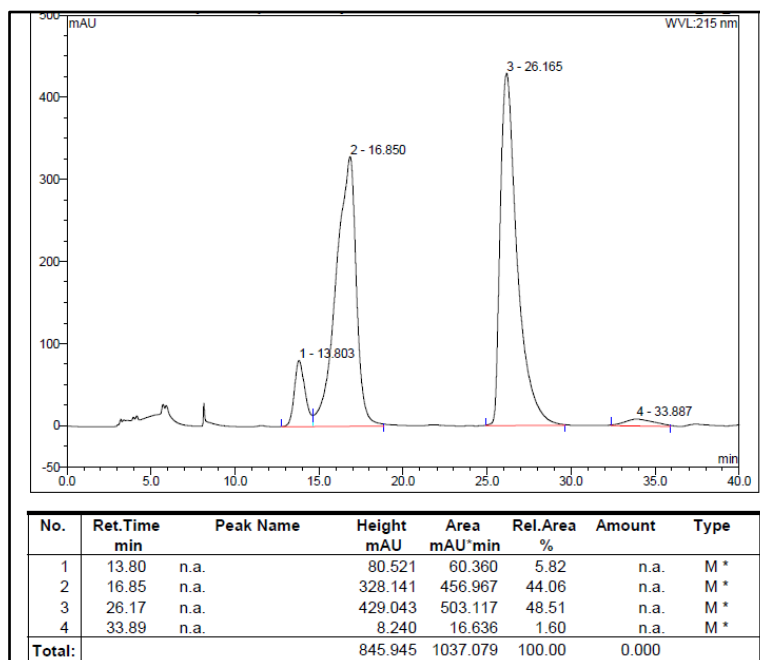
36.000 motor = off
      End

```

A3: Passerini compound 14 calculations



Example: At 20 min Lipase 6Y



From HPLC data:

Peak area % of Product 1: 5.82

Peak area % of Product 2: 1.60

Total peak area of Products: 5.82 + 1.60 = 7.42

Ratio of Product 1: 5.82/7.42 = 0.784

Ratio of Product 2: 1.60/7.42 = 0.216

ee_{PRODUCT} = 0.784 – 0.216 = 0.568 (56.8%)

Peak area % of Substrate 1: 44.06

Peak area % of Substrate 2: 48.51

Total peak area of Substrates: 44.06 + 48.51 = 92.57

Ratio of Substrate 1: 44.06/92.57 = 0.476

Ratio of Substrate 2: 48.51/92.57 = 0.524

$ee_{\text{SUBSTRATE}} = 0.524 - 0.476 = \mathbf{0.048 (4.80\%)}$

From equation 1:

Conversion = $ee_s / (ee_s + ee_p) = 0.048 / (0.048 + 0.568) = \mathbf{0.0779 (7.79\%)}$

Equation 2:

$$E = \ln[1 - c(1 + ee_p)] / \ln[1 - c(1 - ee_p)]$$

$$= \ln[1 - 0.0779(1 + 0.568)] / \ln[1 - 0.0779(1 - 0.568)] = \ln(0.8779) / \ln(0.9663) = \mathbf{\underline{\underline{3.80}}}$$

Equation 3:

$$E = \ln[(1 - c)(1 - ee_s)] / \ln[(1 - c)(1 + ee_s)]$$

$$= \ln[(1 - 0.0779)(1 - 0.048)] / \ln[(1 - 0.0779)(1 + 0.048)] = \ln(0.8779) / \ln(0.9663) = \mathbf{\underline{\underline{3.80}}}$$