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On the Versatility of Microwave-Assisted Chemistry

Exemplified by Applications in Medicinal Chemistry, Heterocyclic Chemistry and Biochemistry

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Abstract
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Today, the demand for speed in drug discovery is constantly increasing, particularly in the iterative processes of hit validation and expansion and lead optimization. Irradiation with microwaves (MWs) has been applied in the area of organic synthesis to accelerate chemical reactions and to facilitate the generation of new chemical entities since 1986. In the work presented in this thesis, the use of MW-mediated heating has been expanded to address three fields of drug discovery, namely hit expansion, chemical library generation and genomics.

In the first project, potential inhibitors of malaria aspartic proteases were designed and synthesized, partly by MW-assisted organic chemistry, and evaluated with regard to their inhibitory efficacy on five malaria aspartic proteases and their selectivity over two human aspartic proteases. The synthetic work included the development of fast and convenient methods of MW-assisted formation of thiazolidines and epoxy esters. Some of the resulting structures proved to be efficacious inhibitors of the aspartic protease that degrades haemoglobin in all four malaria parasites infecting man. No inhibitor affected the human aspartic proteases.

Expedient, two-step, single-operation synthetic routes to heterocycles of medicinal interest were developed in the second and third projects. In the former, the use of a versatile synthon, Ph_3PCCO, provided α,β-unsaturated lactones, lactams and amides within 5–10 minutes. In the latter project, saturated lactams were formed from amines and lactones in 35 minutes, in the absence of strong additives. These two MW-mediated protocols allowed the reduction of the reaction time from several hours or days to minutes.

In the fourth project, a fully automated MW-assisted protocol for the important enzyme-catalysed polymerase chain reaction (PCR) was established. In addition, the PCR reaction could be performed in unusually large volumes, 2.5 mL and 15 mL, with yields corresponding to those from conventional PCR. Good amplification rates suggested that the thermophilic enzyme, Taq polymerase, was not affected by the MW radiation.

Keywords: microwave-assisted chemistry, malaria, aspartic protease, plasmepsin, PfPM4, PmPM4, PoPM4, PvPM4, inhibitor, unsaturated lactone, unsaturated lactam, unsaturated amide, lactam, Bestmann's ylide, ionic liquid, polymerase chain reaction, thermophilic enzyme

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Tanken och känslan skall helst vara förenade.
/.../ Det problematiska är att medan tanken
nästan alltid alstrar känslor, är det långt
ifrån självklart att känslan alstrar tankar.

Kristina Lugn, 2006

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Orrling, K.M., Marzahn, M., Gutiérrez-de-Terán, H., Åqvist, J., Dunn, B.M., Larhed, M.
α-Substituted Norstatines as the Transition-State Mimic in Inhibitors of Multiple Digestive Vacuole Malaria Aspartic Proteases
Submitted.

II Westman, J., Orrling, K. (**2002**)
Cascade Synthesis with (Triphenylphosphoranylidene)-ethenone as Versatile Reagent for Fast Synthesis of Heterocycles and Unsaturated Amides under Microwave Dielectric Heating
Comb. Chem. and High Throughput Screen., 5 (7), 571-574.

III Orrling, K.M., Xiongyu, W., Russo, F., Larhed, M. (**2008**)
Fast, Acid-Free, and Selective Lactamization of Lactones in Ionic Liquids
J. Org. Chem., 73 (21), 8627-8630.

IV Orrling, K., Nilsson, P., Gullberg, M., Larhed, M. (**2004**)
An Efficient Method to Perform Millilitre-Scale PCR Utilizing Highly Controlled Microwave Thermocycling
Chem. Commun., 790-791.

Contents

Abbreviations

A	adenine
ACT	artemisin-based combination therapy
Ala	alanine
aq	aqueous
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BACE-1	β-site amyloid precursor protein cleavage enzyme
bmim	1-butyl-3-methylimidazolium
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
bp	base pair(s)
C	cytosine
CatD	cathepsin D
CE	capillary electrophoresis
CelB	*β*-glucosidase
C_T	cycle threshold
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DCE	dichloroethane
DDT	1,1,1-chloro-2,2-di(4-chlorophenyl)ethane
DHFR	dihydrofolate reductase
DHPR	dihydropteroate reductase
DIEA	*N*,*N*-di-*iso*-propyl-ethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
Dmt	5,5-dimethylthiazolidine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DV	digestive (food) vacuole, of the *Plasmodium* species
EC_{50}	inhibitor concentration resulting in 50% reduced parasite/virus growth
EDC	*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride
equiv.	equivalents
Et	ethyl

EtOAc	ethyl acetate
FO	fibre-optic
FP	falcipain (malaria cysteine protease)
G	guanine
GC	gas chromatography
GE	gel electrophoresis
Gln	glutamine
Glu	glutatamatic acid
Gly	glycine
HAART	highly active antiretroviral therapy
HAP	histo-aspartic protease
HATU	2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV	human immonudeficiency virus
HIV PR	human immonudeficiency virus type 1 protease
His	histidine
HOBt	1-hydroxybenzotriazole
HPLC	high pressure liquid chromatography
HTS	high-throughput screening
IC_{50}	inhibitor concentration resulting in 50% reduced enzymatic activity
*i*Hex	*iso*-hexane
IL	ionic liquid
Ile	isoleucine
IR	infrared
K_i	dissociation constant for inhibitor binding *or* inhibition constant
LC	liquid chromatography
Leu	leucine
LIE	linear interaction energy
Lys	lysine
MAOS	microwave-assisted organic synthesis
*m*CPBA	3-chloroperbenzoic acid
MD	molecular dynamics
Me	methyl
Met	methionine
MS	mass spectrometry
MW	microwave
*n*Hex	*normal*-hexane
nLeu	nor-leucine
NMR	nuclear magnetic resonance
o	*ortho*-position
PCR	polymerase chain reaction
PDB	RCSB protein data bank

Pen	penicillamine
Pf	*Plasmodium falciparum*
Pfu	*Pyrococcus furiosus*
Ph	phenyl
Phe	phenylalanine
PM	plasmepsin (malaria aspartic protease)
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
Pol	DNA polymerase
Pro	proline
PTSA	*para*-toluene sulphonic acid
Pv	*Plasmodium vivax*
PyAOP	(7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PyBOP	(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
quant.	quantitative
RP	reversed-phase
RT	room temperature
rt-PCR	real-time polymerase chain reaction
SAR	structure–activity relationship
sat.	saturated
Ser	serine
SP	straight-phase
T	thymine
Taq Pol	*Thermus aquaticus* DNA polymerase
Thr	threonine
temp.	temperature
Tol	toluene
TS	transition state
Tyr	tyrosine
UV	ultraviolet
Val	valine
WHO	World Health Organization

1 Introduction

1.1 The Drug Discovery Process

The introduction of a new drug onto the market is a complex process including many steps.[1] It can be divided into three main phases, as illustrated in Figure 1:

1. Drug discovery – from therapeutic concept to drug candidate molecule.
2. Drug development – from drug candidate to registered product.
3. Commercialization – from product to therapeutic applications to sales.

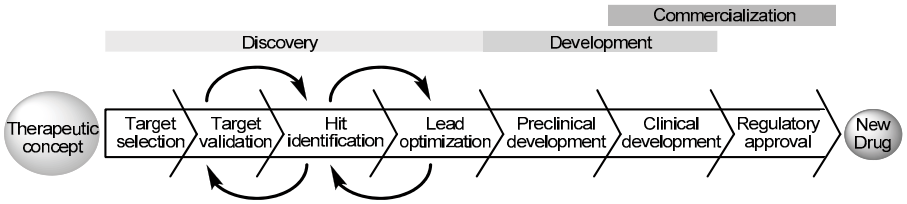


Figure 1. *Schematic summary of the drug development process.*

There are three general trends during the course of the development of a new drug: 1) the number of molecules considered decreases (approx. 10^5-10^7 in early screening to 1 in late clinical trials);[1] 2) the amount of biological data for each compound increases; and 3) the cost increases with time. The total cost of launching a new antimalarial drug has been estimated to be US$ 300 million,[2] which is low compared with at least US$ 800 million for the average drug indication..[3]

Modern drug discovery is a highly interdisciplinary iterative process that precedes the entry in clinical trials of the candidate drug (Figure 2).[1]

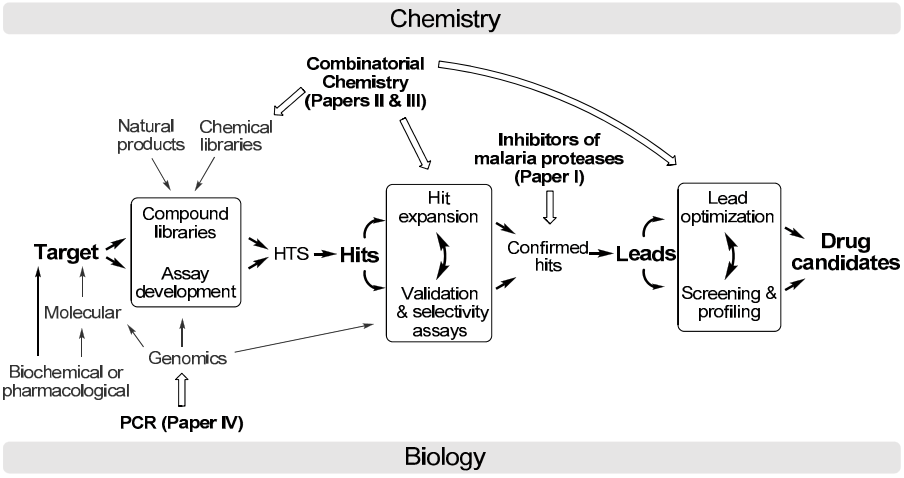


Figure 2. *Typical pathways in the discovery process of a synthetic drug via high-throughput screening, (HTS). The hollow arrows indicate stages where the results of the studies described in this thesis could have an impact.*

The results of the studies described in this thesis are expected to be useful mainly in the early stages of the drug discovery process (Figure 2). The inhibitors of malaria proteases investigated in this work (Paper I) are examples of a hit expansion series that provides new information about the target enzymes in the attempt to produce lead compounds. The synthetic methods described in Papers II and III could be applied to generate entries in a chemical library, for the synthesis of analogues to establish structure–activity relationships (SARs), or to optimize a lead compound in order to obtain a more attractive chemical, pharmacological toxicological or pharmaceutical profile. Paper IV describes an alternative technology which is applied to the polymerase chain reaction (PCR). PCR is a tool used in innumerable aspects of biochemistry, genomics and proteomics, for example, the characterization of the genome, expression of drug target proteins, protein sequence comparisons and to generate mutations in order to obtain proteins that crystallize.

The drug target is generally a protein which is inflicted in the disease, e.g. a receptor, an enzyme, a transport molecule or an ion channel. Once such a biomolecule has been identified, its suitability as a drug target must be evaluated based on: validation, selectivity, biochemical properties, assay development and, in the case of infectious diseases, resistance emergence.[2,4] Target validation, by molecular, biochemical and genomic tools, is of the utmost importance and must be continued throughout the drug discovery process. If a characterized compound exerts an effect on the drug target, ligand-based drug design strategy is possible. If the target amino-acid sequence or, even better, the three-dimensional structure is known, structure-based drug design is facilitated.

The criteria of the *hit*, *lead* and *candidate drug* molecules depend on the targeted disease.[2,5] In general terms, a hit has a pre-defined level of potency *in vitro* and the chemical structure is known. A lead demonstrates potency *in vitro* and *in vivo* in a relevant animal model, is chemically tractable, patentable, selective, exhibits a clear SAR, and complies with defined physicochemical criteria, such as the Lipinski Rule of 5.[6] A drug candidate should have an activity comparable to or exceeding that of standard drugs, if available, and acceptable metabolic, pharmacokinetic and safety pharmacology profiles. Its synthetic scale-up should also be feasible and its mode of action should preferably be understood.

The availability of direct synthetic methods for quick and robust generation of new, tuneable core structures from commercially available reactants is vital for efficient progress in drug discovery and is of great value in all aspects of medicinal chemistry.[7]

1.2 Microwave-Assisted Drug Discovery

Traditionally, heat has been transferred to chemical reaction mixtures by heat transfer from the open flames of Bunsen burners or from heat jackets, oil, sand or water baths. In the late 1970s, inorganic and analytical chemists investigated the possibility of using microwave (MW) irradiation to promote chemical reactions. The first experiments were performed in domestic microwave ovens and suffered from poor controllability and reproducibility. The method was thus considered capricious and unsafe. Similar procedures were used in the first experiments with organic chemical transformations in 1986.[8,9] Since then, MW-assisted organic synthesis (MAOS) has become increasingly popular, especially after the introduction of MW reactors dedicated to organic synthesis. Today, it is a generally accepted tool in organic chemistry, as evidenced by the large number of published review articles[10-23] and books.[24-26] The frequently ascribed advantages to MAOS are:

- shorter reaction times,
- higher yields,
- improved purity,
- greater reaction diversity,
- less amounts of reagents,
- less reactive reagents,
- less amounts of solvents,
- lower energy requirement.

The reduction in the reaction time is attributed to the higher temperature achieved with MW irradiation. Basically, it is the consequence of the Arrhenius equation, $k = A\ e^{(-\Delta G/RT)}$, which states that the reaction rate is exponentially proportional to the temperature.[26] The steeper temperature–time

curve, in combination with the homogeneous temperature profile in the sample, is believed to provide cleaner processes, with and fewer and lower amounts of side products, and to prevent thermal decomposition of substrates and products.

Microwaves are electromagnetic radiation in the frequency region from 0.3 to 300 GHz, i.e. between infrared radiation and radio waves (Figure 3).[24,26] The corresponding energy per MW photon is 10^{-6} to 10^{-3} eV. MW heating equipment, domestic as well as scientific, has been assigned a frequency band of 2.45 (±0.05) GHz, corresponding to a wavelength of 12.2 cm, in order to avoid interference with other MW applications, such as radar and telecommunication. Nonetheless, Bluetooth communication is mediated at the same wavelength.

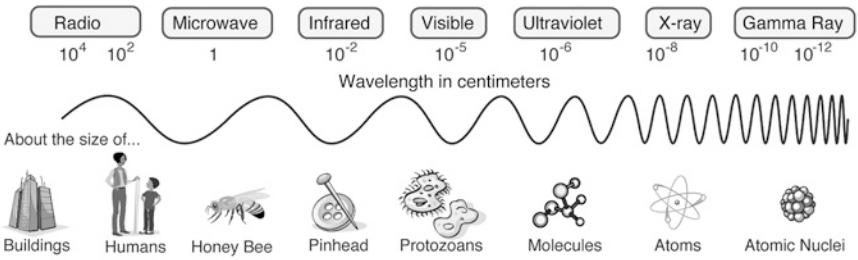


Figure 3. *The electromagnetic spectrum.*

Apart from the applications discussed below, in Section 1.2.2, MW-assisted reactions have been used in other stages of the drug discovery process.[27] The reduced reaction time is an obvious advantage in the preparation of short-lived radiopharmaceuticals used, for example, in target discovery and screening.[28-30] MW-mediated extraction has been found beneficial in the isolation of new chemical entities from plant, fungal and animal extracts.[31] The progress in the MW-assisted production of pharmaceuticals was recently reviewed by Wong.[32]

1.2.1 Microwave Heating Mechanisms

Instead of transferring heat via the reaction vessel, MWs heat the sample mainly by two different mechanisms.[26] Under the influence of an oscillating electromagnetic field, a dipole will rotate so as to align with the field. Energy will arise due to friction of the colliding rotating dipoles (Figure 4a). This phenomenon, named called dipole oscillation, explains why polar solvents, e.g. water and *N*,*N*-dimethylformamide (DMF), can be heated in a MW oven, but not non-polar solvents, such as benzene.

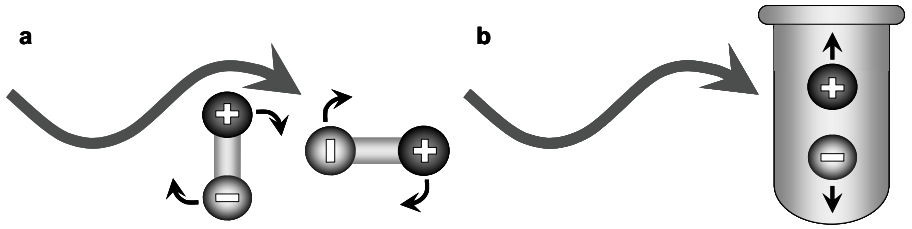


Figure 4. *Microwave heating mechanisms in a liquid, where kinetic energy is transformed into heat by friction. a) Heating by dipole oscillation. The microwave irradiation makes the dipole rotate. b) Heating by ionic conduction. The microwave irradiation will cause ions to move with or against the field depending on charge.*

The second mechanism, ionic conduction, requires the presence of ions in the medium. The ions, being charged particles, will move along with or opposite to the direction of the electric field vector (Figure 4b). As the ions travel through the liquid, collisions will occur and kinetic energy will be converted into heat. Hence, the ion concentration in the reaction mixture is strongly related to the heat absorption. Ionic liquids can thus be added to the reaction mixture to take advantage of the conduction heating phenomenon (see Section 4.3.1).

Non-polar, non-ionic solvents are essentially MW transparent. In such a solvent, only the reactants will be affected by the electromagnetic field and will dissipate energy to the solvent and not vice versa. Hence, the choice of solvent is of great importance. Different solvents or media can be compared by their energy dissipation factors, or loss angles, often expressed as the tangent, $\tan \delta = \varepsilon''/\varepsilon'$, where ε' is the real part of the relative permittivity (the dielectric constant), and ε'' the loss factor.[10,33] Tan δ is a measure of the ability of the irradiated substance to absorb MW irradiation and convert the energy into heat, and is related to the polarity of the compound.

Scientific and domestic MW-heating equipment differ mainly in the design of the irradiation cavity; the former being so-called single-mode instruments while the latter are multimode instruments. The irradiation source, the magnetron, in a household MW oven produces an oscillating electromagnetic beam that is reflected by the walls of the compartment, yielding a complex three-dimensional pattern of standing electromagnetic waves with several different modes, i.e. the mathematical description is the sum of more than one eigenvalue. Hot spots arise at wave maxima and cold spots at minima, as illustrated in Figure 5a.[33,34] The spots are of the same dimension as the wavelength, centimetres, and do not occur at the same locations. Thus, the heating entails the risk of significant variations.[34,35]

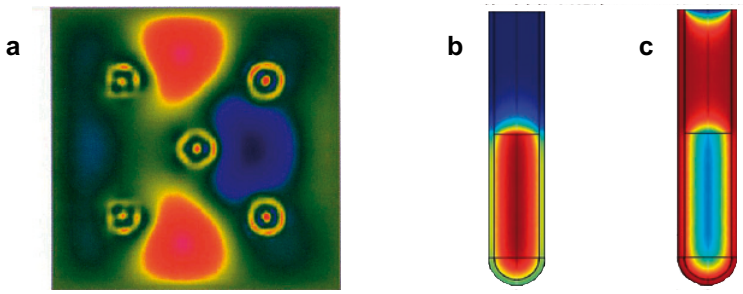


Figure 5. *The simulated temperature patterns produced in a) a multimode microwave cavity showing hot spots (red areas) and cold spots (blue areas), b) a microwave-irradiated sample in a single mode instrument and c) a sample heated in an oil bath* [36]

In a single-mode instrument, the single standing wave that passes through the sample has only one eigenvalue. Hence, the resulting electromagnetic field is, theoretically, uniform within the sample vial and the temperature gradient will be small (as illustrated in Figure 5b). However, the dimensions of the vial must be much smaller than the MW wavelength, i.e. 12.2 cm. A sample of similar size will have hot walls and a much poorer heat distribution if heated in an oil bath (see Figure 5c). In a highly controllable instrument, a device such as a deflector enables the phase of the incoming irradiation to be modified. By changing the geometry of the irradiation cell by moving the deflector, the instrument can be adapted to transfer the optimal amount of heat into various solvents.[26] All MW-assisted procedures performed during this work were conducted in single-mode MW instruments dedicated to organic synthesis.

1.2.2 Temperature Measurements during Microwave Irradiation

The measurement of the temperature of reaction samples during MW irradiation is complicated by the presence of a strong electromagnetic field. Three main methods are used: a shielded thermocouple, an internal fibre-optic (FO) probe or an external infrared (IR) pyrometer.[10,13,37] The advantages and disadvantages of the methods are summarized in Table 1. Today, most commercial scientific MW instruments are equipped with a built-in IR pyrometer. In addition, some producers also market a supplementary fibre-optic probe, which is considered to be the most accurate device. The thermosensitive phosphorescent sensor of a fibre-optic probe can either be based on phosphor or gallium arsenide. Less successful attempts have also been made to measure temperature with a thermometer relying on the expansion of *p*-xylene.[11]

Table 1. *Comparison of different devices for temperature measurements during microwave irradiation.*[13,37]

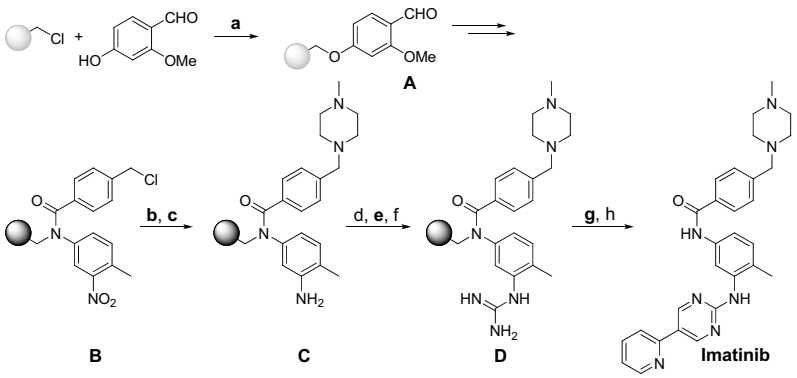
Device	Advantages	Disadvantages
Thermocouple	*In situ* device Cheap Large temperature range Robust	Shielding is required Bulky, due to the extensive shielding Too big for single-mode MW cavities
Fibre-optic probe	*In situ* device Reliable Accurate Small	Fragile Expensive <330–450 °C
IR pyrometer	Large temperature range > 1000 °C Robust	External, i.e. reads the temperature of the outer surface of the vial Calibration required Precise alignment required

Efficient stirring is required for accurate measurement and control of the bulk temperature. In a recent publication, it was reported that the temperature, measured simultaneously by three FO probes, differed by more than 30 °C from top to bottom when the sample, the pure solvent *N*-methylpyrrolidone, was not stirred during heating. In comparison, the variation in temperature was less than 6 °C under stirring.[38]

The existence of a specific non-thermal MW effect has been discussed extensively,[39-41] but has been rejected in the context of organic chemistry and ascribed to the failure to perform accurate temperature measurements.[21,38,42,43]

1.2.3 Microwaves and Medicinal Chemistry

The main advantage of MAOS is the reduction in reaction time. As a result, fewer man-hours are spent on the identification and optimization of reaction parameters, and the total time spent on the synthesis of each test structure can be decreased. In the quest for new chemical entities suitable for chemical libraries for HTS, MAOS has proved to be a valuable tool for the rapid synthesis of complex structures.[26,44-48] In the iterative, and often time-consuming, process of lead optimization, the time required to produce compounds can be reduced by replacing conventionally heating with MW irradiation. Many examples of the MW-assisted production of biologically active and drug-like compounds have been given in the literature.[27,46,49-57] For instance, five steps in the solid-phase synthetic route to the antileukaemic agent imatinib (Gleevec®) are MW-accelerated (Scheme 1).[58] The two most important improvements were, 1) the guanylation of the aniline of **C**, where MW heating resulted in higher yield and purity, and 2) the cyclization of the guanidine group of **D** in the last step prior to cleavage from the resin, which was achieved in 50 min compared to 20 h with conventional heating.



Scheme 1. *Microwave-accelerated, solid-phase synthesis of imatinib (MW-assisted steps indicated by bold arrows).* *Reagents and conditions:* ***a)*** *NaH, DMF,* ***MW****, 120 °C, 5 min;* ***b)*** N-*methylpiperazine, DIEA, DMF,* ***MW****, 100 °C, 5 min;* ***c***) *$SnCl_2$, DMF,* ***MW****, 100 °C, 5 min; d) bis-(*N-*alloc)methylthiopseudourea, $HgCl_2$, NEt_3, DMF, 0 °C, 10 min, then* ***e)*** ***MW****, 80 °C, 5 min. f) $Pd(PPh_3)_4$, $PhSiH_3$,CH_2Cl_2, 1 h;* ***g)*** *3-dimethylamino-1-pyridin-3-yl-propenone, $PhNO_2$, DBU,* ***MW****, 120 °C, 50 min; h) CF_3COOH/CH_2Cl_2, (1/9) 1 h.*[58]

Synthetic routes including a single MW-assisted step to other active pharmaceutical ingredients, such as sildenafil (Viagra®),[59] fluoxetine (Prozac®),[60] duloxetine (Cymbalta®),[60] Betaxolol (Betoptic S®)[61] and modafinil (Modiadal®),[62] have also been reported.

1.2.4 Microwaves and Heterocyclic Chemistry

Heterocycles, i.e. cyclic organic compounds that incorporate one or more heteroatoms in their ring structure, are of the utmost importance in drug discovery. Almost all drug molecules derived or developed from biologically active natural products are heterocycles. Traditionally, the many formations of heterocyclic compounds have required long reaction times at high temperatures, and are thus suitable for MW-assisted protocols. A plethora of heterocycles of various sizes, numbers and types of heteroatoms have been realized by MW-assisted pericyclization,[63] cyclocondensation,[63] multicomponent cascade and tandem reactions and other synthetic routes.[56,64,65] Some early discovered heterocyclic reactions have experienced a revival as a consequence of MW-mediated synthetic procedures. Typical examples are the Fisher indol-synthesis, reported by Villemin *et al.* in 1989,[66] and the extensive work on the Biginelli reaction performed by Kappe.[67]

1.2.5 Microwaves and Biochemistry

MW-mediated heating has found several applications in biochemistry. The first application was the deliberate deactivation of enzymes, particularly in the food industry.[68] In 1994, Jhingan filed a patent on MW-enhanced enzyme-catalysed digestion of DNA, representing the first example of an intentional activation of an enzymatic process by MW irradiation.[69]

On the fringe of organic chemistry, peptides and proteins has been successfully synthesized under conditions similar to those in MAOS.[12,27,70,71] In some interesting cases, the same solvent bulk temperature was measured in the MW-experiment as in the conventionally heated sample, although the outcome was different, for example, higher or lower yields, purity and extent of racemization.[71] Biomolecules are large, and have defined secondary and tertiary structures with a considerable dipole moment,[72] and will thus respond differently to MW irradiation from molecules with a low molecular weight. A biomolecule is less likely to align and realign with the oscillating electromagnetic wave.[33] but parts of it, for instance the highly polarized amide bond, will be affected by the irradiation. Thus, the secondary or tertiary structure of the biomolecule can be altered, with either a beneficial or detrimental outcome.[68,73]

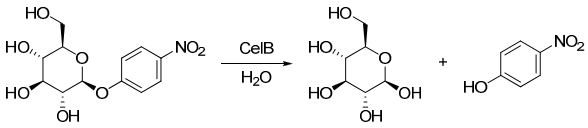
Within the field of proteomics, MW-assisted chemical proteolysis for peptide sequencing and characterization of protein modification has attracted growing interest.[71,74] Moreover, a number of papers have reported MW-assisted proteolytic enzymatic digestion (MAPED).[71,75-78] Recently, Sandoval et al. described a rapid tool for the analysis of post-translational glycosylation defined by a MW-heated method for *N*-deglycosylation by PNGase F.[79] A successful MW-assisted PCR protocol, catalysed by the thermophilic DNA polymerase from *Thermus aquaticus* (Taq Pol) was published in 2003 (this is further discussed in Section 5.1).[80]

Several other chemical transformations have been catalysed by enzymes using MW irradiation and have been presented in a couple of reviews.[68,73] In 1996, several independent groups reported enzyme-catalysed organic-chemical reactions subjected to MW irradiation.[81-84] Recent contributions are the synthesis of modafinil,[62] the synthesis of citronellyl acetate,[85] and enzymatic chiral resolution of alcohols.[86,87] Most of the reported procedures were conducted in organic solvents or under dry conditions, i.e. the enzymes were immobilized on solid supports.

By far the most common enzymes in bio-organic chemistry are lipases, catalysing ester hydrolysis, esterification and various types of transesterification, *N*-acylation, *β*-lactam ring opening, azide reduction and the preparation of chiral amides.[68,73] Glucosidase, chymotrypsin and subtisilin are other enzymes that have been employed in MW-assisted biochemical transformations.

In most of these cases, the enzymatic activity was enhanced upon irradiation with MWs. The improved turnover rate has generally been attributed to non-thermal MW effects. To the best of the author's knowledge, only one report describes how MW radiation alone, independently of a thermal heating effect, causes an alteration in a protein. In 2008, Young et al. showed how the activity of the enzyme *β*-glucosidase (CelB) from the hyperthermophilic archaeon *Pyrococcus furiosus* (Pfu) increased by more than four orders of magnitude during 300 W of MW irradiation.[88] The triplicate samples were simultaneously cooled with a cryogenic fluid at –60 °C preventing the

temperature from exceeding 40 °C, at which Pfu CelB demonstrates minimal catalytic activity, being far below its optimal temperature (110 °C). Pfu CelB cleaves exoglycosidic bonds in both natural and synthetic substrates, for example, the one used in the study by Young et al. (Scheme 2).



Scheme 2. *MW-induced enzymatic cleavage of a glycosidic bond.*

The area of MW-heated enzymatic chemistry and biochemistry is still underdeveloped compared with MAOS and the number of research groups that are active in the field is limited. The uncertainties concerned with the effects of MW irradiation on enzymes and the lack of explanation of the apparent specific non-thermal MW effects make biochemists hesitant to apply the technique. Although lyophilization is an accepted and trivial method of concentrating and drying proteins, and enzymatic catalysis in ionic liquids, which has attracted a great deal of attention during recent years, biochemists tend to view enzymes as too fragile to be subjected to MW irradiation. MW-assisted enzymatic catalysis may become an established tool in biochemistry with time, bearing in mind that the first domestic MW oven was designed in 1945, but did not become a common feature in American homes until the 1970s, and the first papers on MAOS were published already in 1986, but most organic chemists were unaware of the methodology before the 2000s. The increase in popularity in both cases was due to the commercialization of dedicated apparatus with improved user control and safety. New MW instruments with better temperature control in the lower temperature range combined with progress in the research on thermophilic and thermostable enzymes will certainly inspire more researchers to perform MW-assisted bio-catalysed chemistry.

1.3 Malaria

1.3.1 The Burden of Malaria

Of all the infectious diseases prevalent in the tropics, malaria is undoubtedly the most serious. In the countries where it is endemic, malaria takes an enormous toll in terms of lives and quality of life as well as the cost to society (Figure 6).[89,90] In many African countries the word ‘malaria’ is synonymous with disease in general. Indeed, several less severe infections share the clinical symptoms of malaria: fevers, chills, rigor and headaches. In typical malaria infections these recur in regular cycles, but the infection is often

atypical and the clinical manifestations are multifaceted. Severe malaria may include cerebral malaria, seizures, hypoglycaemia, acute renal failure, acidosis and cardiovascular collapse.[91,92]

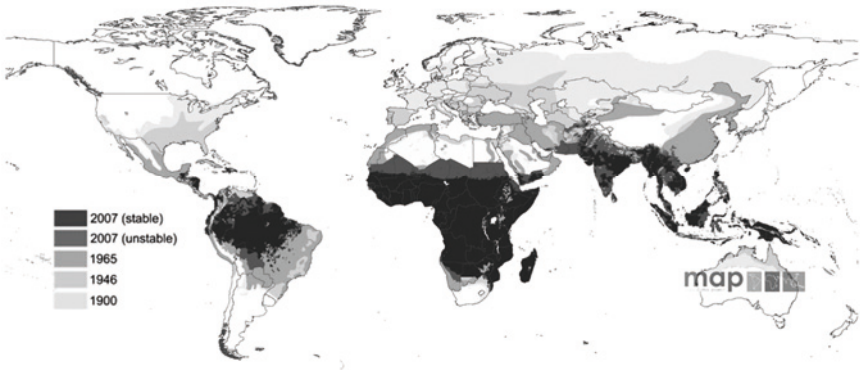


Figure 6. *Malaria is endemic throughout the tropics, but the disease was once widely distributed in the temperate regions.*[93-95]

Over 3.3 billion people live under the threat of being infected with malaria, according to the World Health Organisation (WHO).[90] In 2006, 247 million cases of malaria were clinically diagnosed, and nearly a million people died. Most of these fatalities (85%) were children under the age of five, most of them living in Africa.[*] This implies that two children die from malaria every two minutes. The second most vulnerable group is pregnant women.

Human malaria is caused by four species of the protozoa family *Plasmodium*, *P. falciparum* (Pf), *P. malariae* (Pm), *P. ovale* (Po) and *P. vivax* (Pv). Pf has been the subject of most scientific investigations. The two most important reasons for this are that Pf is responsible for the vast majority of cases of severe malaria and the greatest number of deaths,[90] and it is the only human-infecting species that can be successfully cultivated *in vitro*.[96] Nonetheless, there is evidence that the commonly occurring Pv infection is not as benign as was once believed.[97,98] Other *Plasmodium* species, especially simian forms, can also infect humans, but their clinical impact is of subordinate importance, as the infections are generally self-healing and are not transmitted to other humans. An exception is *P. knowlesi* which has been found to cause fatalities in South-East Asia.[99]

Malaria is transmitted from an infected human by the female mosquitoes of the family *Anopheles*. Combating transmission via the vector has been a major part of programmes aimed at the eradication of malaria for instance the World Health Organisation (WHO) Malaria Program (1955–1969), Roll Back Malaria Partnership (1998–ongoing) and the WHO Global Malaria Program.[100].

[*] This implies that two children die from malaria almost every minute.

The history of human malaria infections is long.[101,102] The name is derived from the Italian “mala aria”, meaning bad air as this was once thought to cause the fever. The renowned Swedish scientist Linnaeus presented a thesis on malaria, in 1735, where he hypothesized that the disease originated from mud particles found in the water in the endemic areas of Sweden.[103] It was not until Laveran discovered the parasite in human blood in 1888 that the true cause was revealed.[104]

Efficient vector control, mainly by 1,1,1-chloro-2,2-di(4-chlorophenyl)-ethane (DDT) spraying, and improved distribution of antimalarial drugs have eradicated malaria from the more prosperous parts of the world (Figure 6). Today, long-lasting insecticidal nets, indoor spraying with insecticides and intermittent preventive treatment in pregnancy along with artemisin-based combination therapy (ACT), are the main tools in the combat against malaria in the developing world.[90]

1.3.2 The Parasite Life Cycle

The life cycle of the *Plasmodium* parasite, schematically illustrated in Figure 7, is complex. It can be divided into a sexual phase in the vector mosquito and an asexual phase in the host animal, i.e., man in the case of Pf, Pm, Po and Pv. The asexual phase is further divided into the pre-erythrocytic stage (also known as the liver stage) and the erythrocytic stage (or the blood stage). The latter is responsible for all clinical aspects of malaria.[91]

The clinically important stages are the ring stage, when detection and differentiation between the infecting species is possible, the trophozoite stage and the subsequent schizont stage, when up to 80% of the haemoglobin in the host erythrocytes is degraded.[105,106] The depletion of haemoglobin induces anaemia in the host, one of the severe clinical manifestations of malaria. The typical malarial fever attacks arise when the merozoites rupture the red blood cells. Pv and Po can develop hypnozoites which remain inactive in the liver, but can cause relapses weeks or months after the initial infection. Most of the symptoms of severe malaria are the consequence of impaired perfusion in capillaries, blocked by swollen or ruptured erythrocytes. The cytoadherence of erythrocytes infected with trophozoites or schizonts, aggravates the situation and lead to a reduced clearance of abnormal erythrocytes in the spleen.[91]

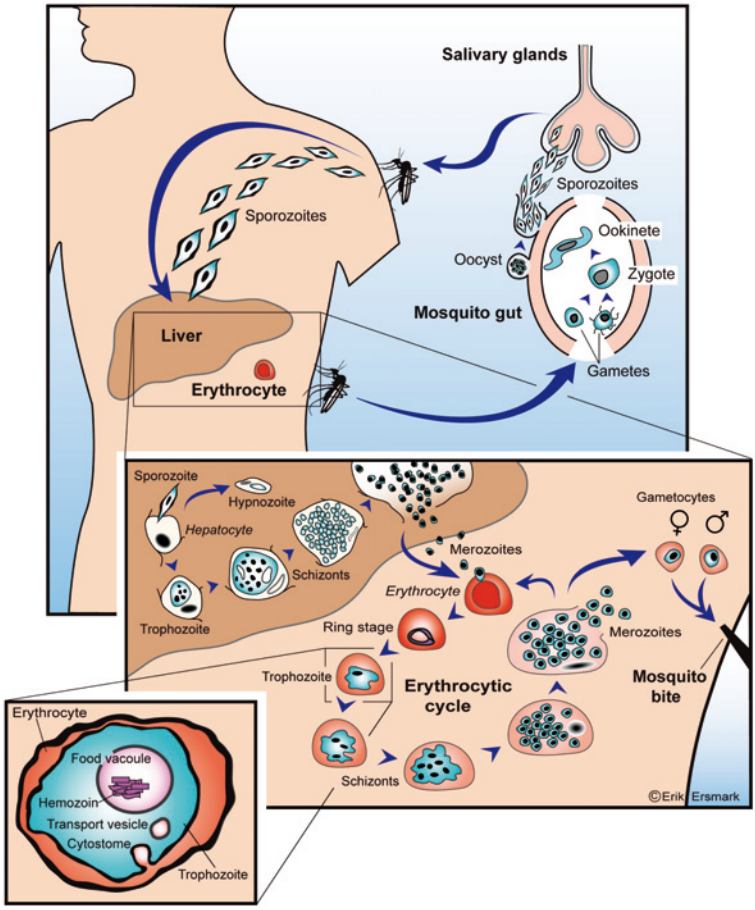


Figure 7. *The life cycle of the malaria parasite in the human host and the mosquito vector. A trophozoite inside an erythrocyte is shown in the insert in the bottom left of the figure.*[107]

1.3.3 Malaria Therapy and Drug Development

Ironically, malaria is probably the only infectious disease that can be treated within three days in a normally healthy induvidual. The prerequisites are early and accurate diagnosis and compliance with the treatment regimen, preferably ACT. An arsenal of drugs can be used in the treatment of malaria, but all are associated with complications, such as severe or frequent side effects and loss of efficacy due to the emergence of resistant parasite strains, as well as high costs (Table 2).[108-111] There is thus an acute need for new therapeutic agents with novel modes of action.[112,113] Most drugs on the market today target either the haem detoxification system or the biosynthesis of purine and pyrimidine bases, for example, by inhibiting dihydrofolate reductase (DHFR) or dihydropteroate reductase (DHPR). However, specific protein targets have not been identified for all existing antimalarial drugs.

Table 2. *Characteristics of the currently most frequently used antimalarial drugs.*[109]

Drug	**Launched**	**Target**	**Side effects**	**Resistance emergence**[113]	**Cost** (US$)/ **treatment**[108]
Quinine	> 1820	Haem detoxification?	**	****	1.35
Chloroquine	1930s	Haem detoxification	***	*****	0.072
Proguanil[a]	1940s	DHFR	*	-	-
Amodiaquine	1940s	Haem detoxification	**	***	0.15
Sulphadoxine-pyrimethamine	1950s	DHPR, DHFR	**	****	0.082
Primaquine[b]	1950s	Unknown	**	***	0.15
Halofantrine	1960s	Haem detoxification	****	**	4.75
Mefloquine	1970s	Haem detoxification	**	***	2.14
Artemisinin derivatives	1970s	Calcium ATPase	*	*[110,111]	2.16–4.20
Ataquanone[b]	1990s	Mitochondrial electron transport	*	***	42

* – ****, Low – high incidence or severity. a) Not used as monotherapy; b) also active against intrahepatic forms. c) Price for the combination therapy with proguanil.

Specific requirements must be considered in the development of drugs against malaria, because of the nature and the geographical distribution of the disease. Pink et al. have defined several requirements for new antimalarial drugs:[2]

- they should be active against Pf,
- they should be effective against drug-resistant parasites,
- they should have an oral formulation,
- the course of treatment should be short (once daily for ≤ 3 days) and
- the cost/treatment should be ≤ US$ 1.

Desirable properties are that they:

- are active against at least Pv, but preferably also Pm, Po and Pk,
- have the potential for combination therapy,
- have the potential for use in unconscious patients,
- have the potential for use in cerebral malaria,
- are suitable for prophylaxis,
- are fast-acting in the relief of symptoms,
- block transmission to mosquito and
- have a good shelf-life in tropical climates.

None of the human malaria parasites (Pf, Pm, Po and Pv) can infect other species, so the development of antimalarial drugs is reliant on animal models infected by alternative species of *Plasmodium*, e.g. the murine forms *P. berghei*, *P. yoelii*, *P. chabaudi* and the simian *P. knowlesi*.[2] Two complications in structure-based drug design are the high concentration of A–T-rich codons in the Pf genome and the abundant low-complexity protein regions, which make gene expression and suppression and protein cloning a chal-

lenge.[114] However, the mapping of the genome[115] has facilitated protein purification and improved the crystallization rate.[116] Several proteins in various parasite organelles, for example the apicoplast, mitochondrion and the digestive vacuole (DV) have been identified as putative drug targets. A number of proteins involved in the cell cycle or found in the membranes or cytosol are also under investigation.[114,117,118]

1.3.4 Haemoglobin Metabolism

The intraerythrocytic parasites have limited capacity for *de novo* amino acid biosynthesis. Therefore, they rely on the catabolism of human haemoglobin for their supply of some amino acids to maintain protein synthesis, homeostasis[106,119] and metabolism.[120] Human haemoglobin is ingested by the parasite and transported to a specialized organelle, the DV*, with a pH significantly lower (pH ~5) than that of the cytoplasm (~7.4) (see the insert in the bottom left of Figure 7).

The haemoglobin is broken down into di- and tripeptides by a cascade of proteolysis reactions performed in the concerted action of a multitude of proteases in the DV.[121-123] In Pf these proteases are: 1) four plasmepsins, i.e. three aspartic proteases, (PfPM1, PfPM2 and PfPM4) and a closely related histo-aspartic protease (HAP, previously called PfPM3), 2) the falcipains, i.e. four cysteine proteases, (PfFP1, PfFP2, PfFP2′ and PfFP3),[124-127] 3) falcilysin, a metalloendopeptidase,[128,129] and 4) a dipeptidyl aminopeptidase (PfDPAP1)[127]. The oligo- and dipeptides are exported to the parasite cytoplasm, where further hydrolyses, involving metalloexopeptidases PfA-M1 and PfA-M17, generate free amino acids.[130-133]

All proteases involved in haemoglobinolysis have been targeted for drug discovery.[114,117,118,134-136] Apart from the plasmepsins (PMs), discussed in more detail in Section 1.3.6, most scientific efforts have been directed towards the falcipains (FPs). The two enzyme families have overlapping functions[123,137,138] and are not ordered as once believed.[139] Both PMs and FPs can accomplish the initial cleavage between Phe33 and Leu34 in the hinge region of haemoglobin and the subsequent cleavage into smaller peptides. Falcilysin is only capable of cleaving smaller peptides into oligopeptides, which are subsequently subjected to proteolysis by DPAP1, furnishing dipeptides. PfFP2 and PfFP3 have been proven to be essential for parasitic growth by studies on knock-out Pf parasites,[140] in contrast to the paralogue PfFP1.[141] Unfortunately, parasite strains resistant to cysteine protease inhibitors have been detected.[142]

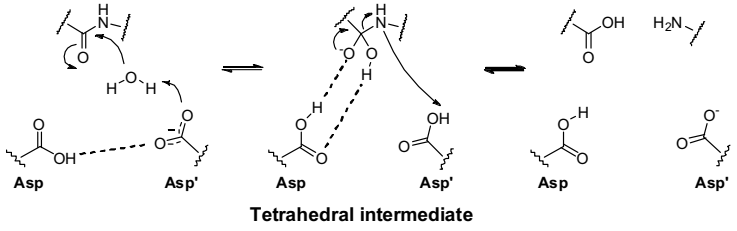
Besides the protein coils, haemoglobin also contains haem, which is released in the degradation process. The ferrous haem is oxidized to ferric haematin, but both are toxic to the parasite. Thus, haematin is polymerized to

* Also known as the food vacuole, corresponding to the lysosome in animal cells.

inert, crystalline haemozoin, which is stored in the DV of the parasite. The detoxification of haem is the target of several antimalarial drugs (see Table 2). Haemozoin is released into the blood upon the rupture of the erythrocyte and colours the faeces and urine of malaria patients dark, hence its alternative name 'malaria pigment'. Haemozoin can be easily detected by microscopic methods, providing an unambiguous diagnosis.

1.3.5 Aspartic Proteases

The first aspartic protease to be targeted for drug development was renin. A great deal of research was performed in the 1970s and 1980s. Although no hypertension drug resulted from this work until very recently,[143] the knowledge accumulated could be applied to new aspartic protease drug targets, including the HIV-1 protease, resulting in 11 approved HIV drugs. An important finding was that efficacious inhibitors could be obtained by replacing the scissile peptide bond by new structural elements mimicking the tetrahedral intermediate in the peptide cleavage mechanism (see Scheme 3).[144] In fact, this transition-state (TS) isostere strategy has been so successful that it has become the general methodology for the design of almost all aspartic protease inhibitors.[145]



Scheme 3. *Proposed catalytic mechanism of substrate hydrolysis by aspartic proteases.*

The substrate/inhibitor side chains (Pn, Pn′) and the corresponding enzyme subsites (Sn, Sn′) are labelled based on their position relative to the scissile bond (Figure 8).[146]

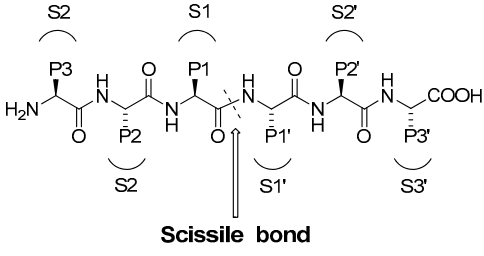


Figure 8. *Nomenclature used for protease substrate residues (Pn–P1 / P1′–Pn′) and their corresponding binding sites in the enzyme (Sn–S1 / S1′–Sn′), according to the Schecter–Berger convention.*[146]

The human genome encodes only for eight functionally active aspartic proteases (black in Figure 9), which are generally expressed at relatively low concentrations.[145,147] This reduces the problem of identifying substrate selective inhibitors of parasite/viral aspartic proteases.

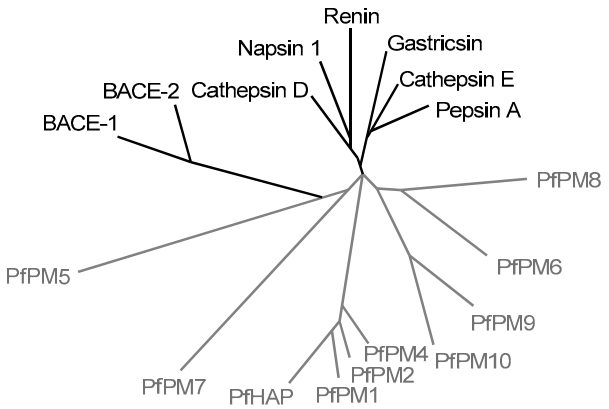


Figure 9. *A dendogram showing the presently known aspartic proteases from human (black) and* P. falciparum *(grey).*[147]

1.3.6 Plasmepsins

The genome of Pf encodes for ten aspartic proteases (PfPM)[115] which belong to the enzyme subfamily A1 (grey in Figure 9).[145] PfPM1,2,4,5,9,10 and PfHAP are expressed in the erythrocytic stage, whereas PfPM6–8 are expressed in the exo-erythrocytic stages.[148] As the known aspartic protease inhibitor pepstatin A has been shown to reduce haemoglobinolysis and parasite growth,[149,150] the PMs in the DV (PfPM1,2,4 and HAP) have been extensively studied,[121,147,148,151-159] yet the role of the other PM enzymes remains uncertain. Although PfPM1 was the first plasmepsin to be discovered,[149] PfPM2 has been the subject of most scientific activity, mainly because it proved to be easier to express, process purify and, not least, crystallize.[160] Hence, the large majority of all published plasmepsin inhibitors have only been tested with regard to their efficacy against PfPM2.[107] Of the four PMs found in the DV of Pf, only the orthologue of PfPM4 is expressed and active in the other human malaria species (PmPM4, PoPM4 and PvPM4).[155,161] Three of the gene disruption studies that have been performed on PfPM show significantly reduced growth rate in the *pfpm4* knockout parasites, compared with the wild-type parasites and parasites lacking *pfpm1*, *pfpm2* and *pfhap*, respectively.[162-164] The failure of the single disruption of *pfpm4* in two other studies, may indicate that PfPM4 has a critical role.[122,123] The quadruple *pfpm1-4* knockout parasites survive, although their vitality is severely impaired.[163]

The RCSB protein databank[165] (PDB) contains 16 structures for PfPM2, including the apo-enzyme (1LF4, 3F9Q), proplasmepsin (1PFZ), the enzyme in complex with pepstatin A (1W6I, 1M43, 1SME, 1XDH), and other inhibitors (1W6H, 2BJU, 1LF2, 1LF3, 1LEE, 1ME6, 1XE5, 2R9B). No structures have been deposited for PfPM1 or PfHAP, but structures for PfPM4–pepstatin A (1LS5),[166] PvPM4–pepstatin A (1QS8),[167] apo-PvPM4 (1MIQ)[168] and PmPM4–inhibitor **KNI-764** (2ANL)[169] complexes have been submitted.

The PM4s, as well as PfPM2, have a bi-lobal topology similar to that of eukaryotic aspartic proteases (Figure 10).[170] The two domains are connected where the catalytic dyad, Asp34 and Asp214, is found. The flap, a flexible β-hairpin structure, lies perpendicular over the binding cleft and locks the substrates/inhibitors in place in the binding pocket.[144] The most important interspecies difference is found in the flexible loop (Val236–Tyr245), further away from the active site.[169]

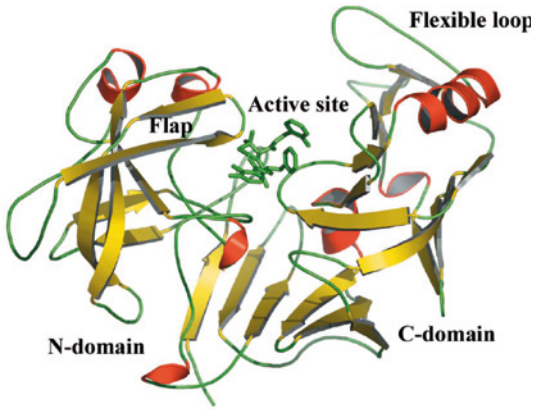


Figure 10. *Ribbon representation of the crystal PmPM4–**KNI-764** complex (2ANL).*[169]

Analysis of the most important amino acid residues at the binding site of the PMs, reveals a particularly high sequence identity between the PM4s (Table 3).[166,167,169,171,172] The interorthologue variations generally imply substitutions of amino acids with similar properties (e.g. Ile75Leu and Thr219Ser). The differences between PfPM2 and the PM4s result in some structural distinction. For example, Met75Ile/Leu exhibits a more constrained S2′ binding site and Val78Gly a less bulky flap loop.

Table 3. *Polymorphisms of the residues in the binding sites of the plasmepsins.*[169,171]

Residue/ Enzyme	**34**	**36**	**37**	**75**	**77**	**78**	**79**	**111**	**114**
PfPM1	Asp	Gly	Ser	**Met**	Tyr	**Val**	Ser	Phe	**Ala**
PfPM2	Asp	Gly	Ser	**Met**	Tyr	**Val**	Ser	Phe	**Thr**
PfHAP	**His**	**Ala**	Ser	Leu	**Ser**	**Lys**	**Ala**	Phe	Phe
PfPM4	Asp	Gly	Ser	Ile	Tyr	Gly	Ser	Leu	Ile
PmPM4	Asp	Gly	Ser	Ile	Tyr	Gly	Ser	Leu	**Leu**
PoPM4	Asp	Gly	Ser	**Leu**	Tyr	Gly	Ser	Leu	**Val**
PvPM4	Asp	Gly	Ser	Ile	Tyr	Gly	Ser	Leu	Ile
Residue/ Enzyme	**131**	**133**	**192**	**214**	**217**	**219**	**290**	**292**	**294**
PfPM1	Leu	Ile	Tyr	Asp	Thr	**Ser**	**Ile**	Val	Leu
PfPM2	Leu	Ile	Tyr	Asp	Thr	**Ala**	**Ile**	**Leu**	**Phe**
PfHAP	Leu	Ile	**Met**	Asp	Thr	**Val**	**Val**	**Ile**	Leu
PfPM4	Leu	Ile	Tyr	Asp	Thr	Thr	Leu	Val	Ile
PmPM4	Leu	Ile	**Phe**	Asp	Thr	Thr	Leu	Val	Ile
PoPM4	Leu	Ile	Tyr	Asp	Thr	**Ser**	**Ile**	Val	Ile
PvPM4	Leu	Ile	Tyr	Asp	Thr	Thr	Leu	Val	Ile

1.3.7 Other Aspartic Proteases in Drug Discovery

Cathepsin D (CatD) is the most closely related mammalian aspartic protease to the plasmepsins. Human CatD (hCatD) and PfPM2 share a 35% sequence identity and an even higher degree of homology at the binding site.[170,173] hCatD is a lysosomal enzyme, thus it is present in almost all types of cells. It has been demonstrated to be essential for survival in knockout mice studies.[174] Therefore, it is of the utmost importance to verify good selectivity of putative inhibitors towards pathogenic aspartic proteases.

The human *β-site amyloid precursor protein cleavage enzyme* (BACE-1)* is responsible for proteolytic activity in the cascade forming the amyloid plaques characteristic of the brains of patients with Alzheimer's disease. Extensive research has been conducted to find efficacious inhibitors of BACE-1.[175] Both BACE-1 and hCatD belong to the same enzyme subgroup (A1) as the plasmepsins.

HIV-1 protease (HIV PR) cleaves the viral polyprotein and is essential for the maturation of viral particles.[176] The approved HIV PR inhibitors are important components in the HIV and AIDS multidrug therapy, known as highly active antiretroviral therapy (HAART).[177]

HIV PR belongs to the A2 subclass of aspartic proteases.[145] It is a dimeric C_2-symmetric enzyme, consisting of two identical subunits, which has been

* Also known as memapsin-2 or β-secretase.

studied in a large number of solved crystal structures.[165] HIV PR has been the target of a number of successful structure-based drug design studies. Despite the approval of 11 anti-HIV drugs, the search continues for new HIV PR inhibitors with 1) improved bioavailability, 2) better efficacy against the many resistant strains, 3) better selectivity profiles with fewer side effects and 4) better pharmacokinetic properties for the development of once-daily pills.

Approved HIV PR inhibitors have been demonstrated to 1) reduce Pf growth,[178-180] 2) be inhibitors of PfPM2,[179,181] and PfPM4,[182] and 3) have an antiplasmodial effect independent of PfPM1–4 inhibition.[183,184] Of the anti-retroviral drugs, lopinavir, ritonavir and saquinavir have the highest general potencies in both cell-based antiplasmodial assays and against the isolated PfPM2 and PfPM4.

1.3.8 Inhibitors of Plasmepsin 4 and other Plasmepsins

Aspartic protease drug discovery has been dominated by two approaches: structure-based drug design and ligand-based drug design. The latter approach is based on the identification of the cleavage site of natural substrates (Table 4), non-natural substrates and investigated inhibitors.[185]

The use of kinetic assays to determine the rates of cleavage of octapeptides revealed that the PM4s preferred Phe in P1, whereas PfPM2 accepted Leu, nLeu and Phe.[172,186] All bulky hydrophobic natural amino acid residues were well tolerated in P1′, with a bias towards Tyr by PfPM4 and PoPM4, towards Phe by PmPM4 and PvPM4 and towards Leu by PfPM2. Small and constrained residues, e.g. Gly and Pro, were not well tolerated. Similar trends were found for the residues in P2 and P2′. Ten octapeptides with a reduced amide as a TS mimic, exemplified by **A** in Figure 11, were designed based on these results, yielding the first inhibitors of all four PM4 involved in human malaria infection ever published.[172,186] Structures similar to **A** were evaluated on PfPM4, PmPM4 and PvPM4, in 1997.[155] Since then, inhibitors have only been assessed on all four PM4s by Janka et al in 2008.[187] In that publication 16 less peptidic, but large inhibitors were evaluated (exemplified by **B** Figure 11).

Table 4. *Comparison of the of PfPM and HIV-PR in natural substrate cleavage sites (*).*[185]

Enzyme	Cleavage site	P3	P2	P1	P1′	P2′	P3′
PM	α33*34	Arg	Met	Phe	Leu	Ser	Phe
	α108*109	Leu	Val	Thr	Leu	Ala	Ala
	α136*137	Thr	Val	Leu	Thr	Ser	Lys
	β32*33	Arg	Leu	Leu	Val	Val	Tyr
HIV-1 PR	p17*p24	Arg	Gly	Tyr	Pro	Ile	Val
	p24*p1	Arg	Val	Leu	Ala	Glu	Ala
	p1*p9	Thr	Ile	Met	Met	Gln	Arg
	p9*p6	Gly	Asn	Phe	Leu	Gln	Ser
	TF*PR	Phe	Asn	Phe	Pro	Gln	Ile
	PR*RevT	Leu	Asn	Phe	Pro	Ile	Ser
	RevT*RN	Glu	Thr	Phe	Tyr	Val	Asp
	RN*IN	Lys	Ile	Leu	Phe	Leu	Asp

TF, transframe protein; PR, protease; RevT, reversed transcriptase; RN, ribonuclease H; IN, integrase

Although the number of inhibitors of PfPM4 is considerably smaller than that to PfPM2, the compounds represent a variety of structural classes (Figure 11).[107] Two of these, **F** and **H**, exhibit good potency against Pf growth in infected erythrocytes and against PfPM4, but not against the PfPM2 paralogue.[188,189] One of the best adaptive inhibitors of all DV-PM paralogues is **KNI-10006** (**D** in Figure 11).[185,190] It is derived from an HIV PR inhibitor programme and exhibits low nanomolar activities against both PfPM2 and PfPM4.[191,192] Unfortunately, it is also a good inhibitor of hCatD, and the *in vitro* antiplasmodial activity (EC_{50} = 6.8 μM) is somewhat disappointing.[185] These two drawbacks of the inhibitor class have only been partially remedied by extensions in the P2 and P3 positions.[193,194]

In order to target the PMs in the DV, many biological membranes have to be passed, both in the host and the parasite. Several PM inhibitors incorporate a basic nitrogen, for example the structures **A**,[186] **B**,[187] **G**[195] and **H**,[107] to trap the compounds in the acidic DV, once they have entered. Another approach is to ensure good membrane permeability by masking polar substituents with steric bulk or intramolecular hydrogen bonding. A successful example of this is the introduction of a tertiary-alcohol scaffold in HIV PR inhibitors of type **I** (Figure 11).[196,197] These inhibitors exhibit excellent membrane penetration rates and the inhibitory efficacies on isolated HIV PR are compable with the one of atazanavir.

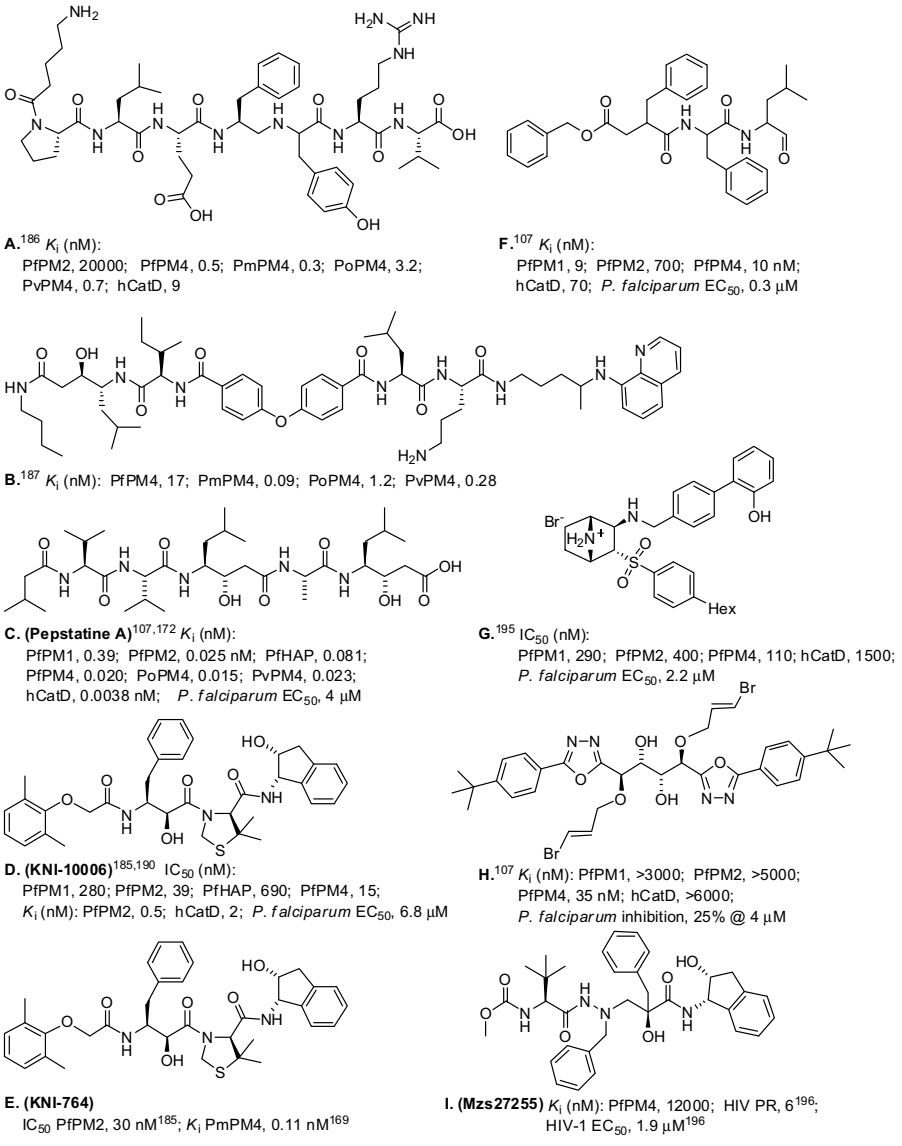


Figure 11. *Examples of various types of PfPM4 inhibitors.* [107]

In most series of aspartic protease inhibitors, a significant difference has been observed in inhibitory efficacy between epimers having the opposite stereochemistry in the TS-mimicking hydroxyl group. The (*R*)-isomer of **I** is almost 400 times less potent than the (*S*)-isomer on HIV PR.[196] On PfPM4, however, the difference is only four-fold, probably due to a less precise fit in the active site of the enzyme.

1.4 Lactams in Medicinal Chemistry

1.4.1 Lactams in Drug Structures

The importance of five-, six- and seven-membered lactams, i.e. cyclic amides, in natural product bioorganic and medicinal chemistry is widely known.[198] The lactam scaffold is found in a number of approved drugs, e.g. the nootropic agent piracetam (Nootropil®), the anticonvulsant levetiracetam (Keppra®), the immunomodulatory drug lenalidomide (Revlimid®), the 5-HT3-antagonist palonosetron (Aloxi®) and the cardiotonic ivabradine (Procoralan®) (Figure 12). Lactams are extensively used as turn-mimetics and rigidified peptidomimetics, for example, in a number of HIV PR inhibitors, for example **J**[199] and BACE-1 inhibitors, for example **K** (see Figure 12).[175]

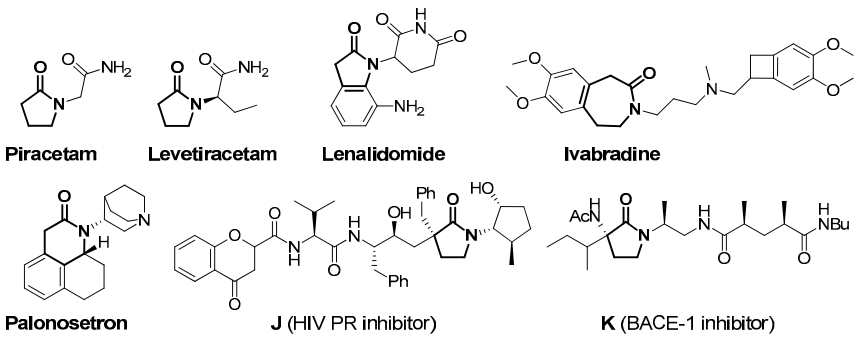


Figure 12. *Approved drugs and aspartic protease inhibitors (**J** and **K**) with a lactam scaffold (indicated in bold).*

The versatility of both acyclic and cyclic *α*,*β*-unsaturated amides as synthetic reagents is exemplified by their ability to undergo epoxidation, halogenation, cycloaddition, Diels–Alder reaction, Michael addition and other 1,4-additions.[200-202] The usefulness of *α*,*β*-unsaturated amides in medicinal chemistry is illustrated by the last step in the synthesis of imatinib (conditions g, Scheme 1).

1.4.2 Synthesis of Lactams

Traditionally, lactams are formed by the following methods.[200-202]

- Cyclization of amino acids, halo amides and unsaturated amides
- Ring opening and closing of lactones by ammonia and amines
- Metal-catalysed hydrocarboxylation of unsaturated amines
- Reaction between imines, Zn and halo esters
- Reaction between imides and phosphoranes
- Addition of ketenes to imines and of enamines to isocyanates
- Ring expanding rearrangements, e.g. Beckmann rearrangement, Belluš–Claisen rearrangement, Schmidt reaction and Stieglitz-type rearrangement
- Oxidation of cyclic tertiary amines

The number of reactions furnishing α,β-unsaturated lactams in few steps is considerably smaller. Nevertheless, a few procedures have proved to be useful, such as cycloaddition of α,β-unsaturated imines and carbonyls,[203-207] ring-closing metathesis reaction of α,β-unsaturated allylamides,[208] reaction between primary amines and dimethoxy dihydrofuran under aqueous acidic conditions,[209] cyclocondensation of 3-alkenamides and aldehydes[210,211] and reaction of isopropylidene-D-glyceraldehyde with a silyloxypyrrole.[212]

1.5 α,β-Unsaturated Lactones in Medicinal Chemistry

1.5.1 α,β-Unsaturated Lactones in Drug Structures

The α,β-unsaturated lactone motif is found in many biologically active structures. Five-membered unsaturated lactones, often referred to as butenolides, are found in the now withdrawn non-steroidal anti-inflammatory cyclooxygenase-2 inhibitor rofecoxib (Vioxx®) and the related firocoxib[Clarcke 2006] (Eqioxx®, Previcox®), which was approved for veterinary use in 2007, as well as the antiarrhythmic cardiac glycoside digoxin (Linoxin ®) (Figure 13). Ascorbic acid (vitamin C) is beyond doubt the most well-known butenolide. Six-membered unsaturated lactones are represented by the recently introduced HIV-1 protease inhibitor tipranavir (Aptivus®), and the photoactive drug methoxsalen (Oxsoralen®) used for the treatment of psoriasis, eczema and vitiligo (Figure 13).

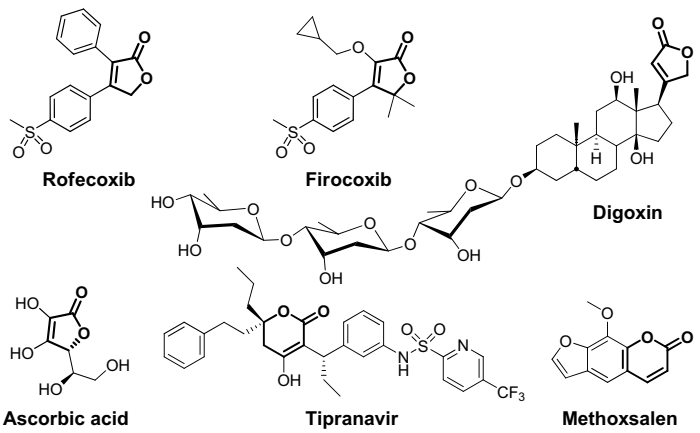


Figure 13. *Marketed drugs with an α,β-unsaturated lactone core (indicated in bold).*

Innumerable natural products encompassing an α,β-unsaturated lactone scaffold with potent biological activity have been isolated and evaluated. Some examples are butyrolactone I,[213] losigamone,[214] manoalide,[215] and triptolide,[216] all of which are γ-lactones. Fostriecin, leptomycin B and ouabain are examples of δ-lactones that have recently attracted attention.[217,218]

Like their N-analogues α,β-unsaturated lactones are important and versatile starting materials and can undergo many types of reactions useful in medicinal chemistry.[201,202] For instance, a butenolide is the precursor of the ditetrahydrofurane moiety of the HIV protease inhibitor darunavir.

1.5.2 Synthesis of α,β-Unsaturated Lactones

Lactones are cyclic esters that can be formed by intermolecular esterification from ω-hydroxy carboxylic acids which may be activated or not.[201,202,219,220] One reaction using 2,4,6-trichlorobenzoyl chloride and 4-dimethylaminopyridine (DMAP), called the Yamagutchi reaction, is a very mild procedure used to form macrolides, i.e. large ring-size lactones, and is common in the area of total synthesis. Alternative procedures for the preparation of lactones are the Baeyer–Villiger oxidative rearrangement of cyclic ketones or the oxidation of cyclic ethers. Several lactamization methods of can be adapted to oxygen-based substrates to yield lactones.[201,202,219,220] However, these methods are generally not applicable in the direct synthesis of α,β-unsaturated lactones for which other strategies have been developed.[219-222] These methods have recently been reviewed.[217] Single-step procedures are few and include hetero Diels–Alder reactions, the Cu-catalysed vinylogous Mukaiyama reaction, Pd-catalysed multicomponent coupling reactions, Keck's annulation, i.e. ring closing metathesis of homoallylic alcohols or esters, and cyclocarbonylation of unsaturated alcohols.[217,221-223]

1.6 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a nature-mimicking technique, allowing a certain region of deoxyribonucleic acid (DNA) to be exponentially multiplied *in vitro*. As the number of identical DNA segments, or amplicons, grows, the DNA sequence of interest can be detected and distinguished from other segments, i.e. the signal from the specific DNA target sequence is amplified. The discovery and use of the PCR has been a true breakthrough in the world of biosciences and its inventor, K. B. Mullis,[224] was awarded the Nobel Prize in 1993.

The importance of the PCR technique can not be overestimated. Increasing the amount of DNA has allowed the use of DNA in forensic investigations, determining genetic relationships and in investigating the presence of hereditary diseases. The PCR technique has also been used to identify and categorize organisms, for the early diagnosis of malignant diseases and for determination of parasite or viral load in, for example, HIV patients.[225] One of the developers of the PCR method, Stephan Scharf, has said that the truly astonishing thing about PCR is that it was not designed to solve a problem, but once it existed,

problems began to emerge to which it could be applied. PCR is a tool that has the power to create new situations for its use and those required to use it.[225]

The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the reaction in a defined series of temperature steps. A typical PCR thermocycle includes the following steps, which are illustrated in Figure 14.[226]

1. **Denaturation**. The hydrogen bonds between the DNA amino bases are broken under the influence of heat. The complementary strands of the DNA molecule dissociate to yield two single-stranded molecules. Melting of the DNA molecule normally occurs at 90–98 °C.
2. **Annealing**. Each end of the target sequence is identified by two specific primers, oligonucleotides of ~20 base pairs (bp). By lowering the temperature new hydrogen bonds can be established between the primers and the complementary region of the single-stranded DNA template. Annealing is normally performed at 50–60 °C.
3. **Elongation**. DNA polymerase attaches deoxyribonucleotide triphosphates (dNTPs) to the primer by creating a phosphate bond between the 3′-hydroxyl group of the primer and phosphate of the dNTP (see Figure 15). The temperature should correspond to that at which the enzyme of choice shows optimal activity.
4. **Repetition**. Steps 1–3 are repeated several times (normally 15–40 times). Provided that the reaction goes to completion and assuming the number of cycles to be 33, the theoretical amplification is $8.6 \times 10^9 = 2^{33}$.

The discovery and application of the thermostable Taq Pol made the technique extremely efficient, as all the steps could be conducted in sealed tube using an automated instrument (called a thermocycler).[227] A typical PCR thermocycle is depicted in Figure 16a.

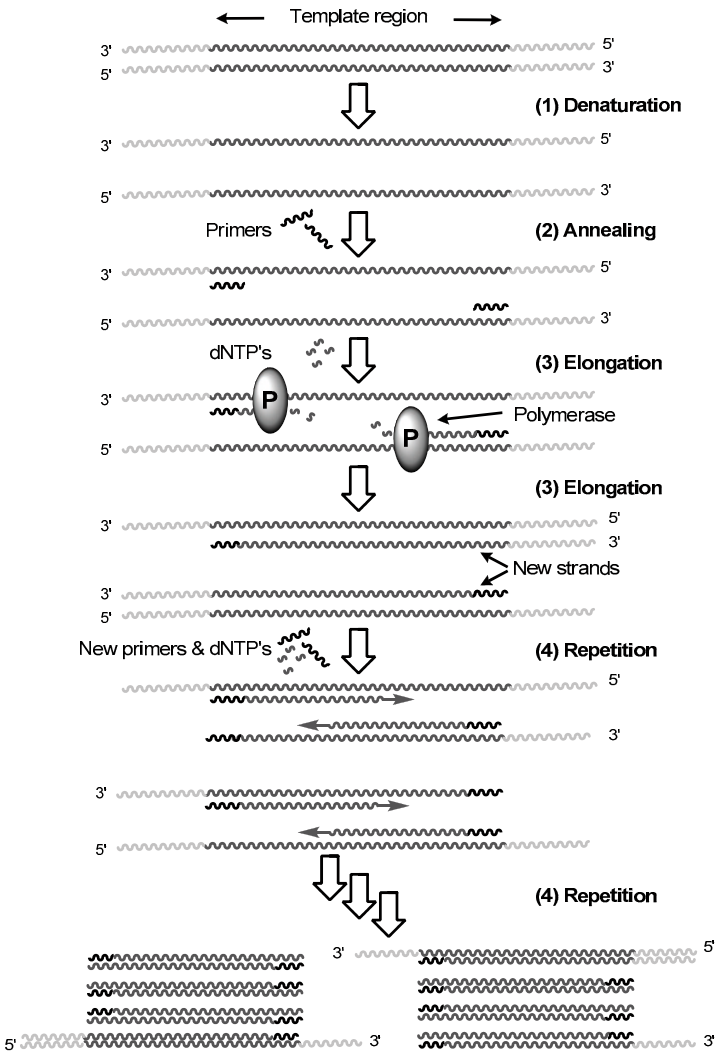


Figure 14. *Schematic illustration of PCR thermocycling. (1) Denaturation, (2) Annealing, (3) Elongation, catalysed by DNA polymerase (P), and (4) Repetition.*

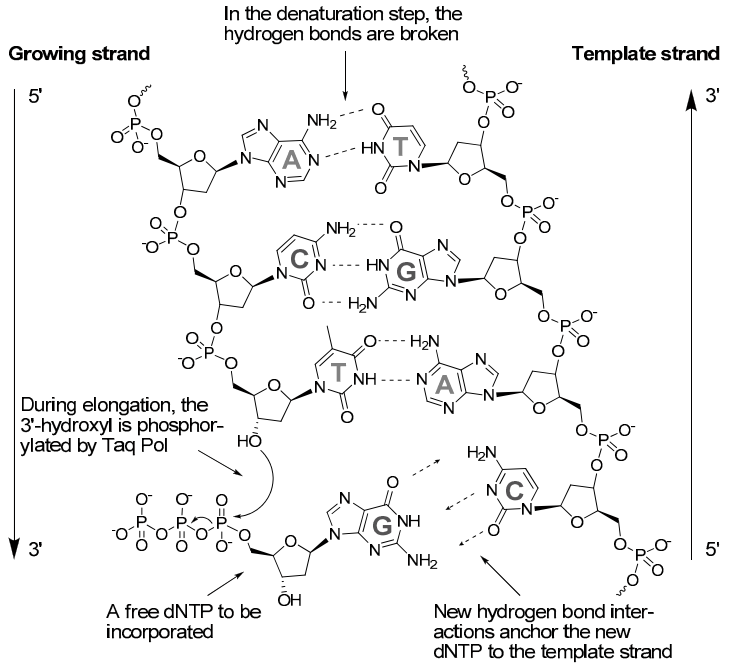


Figure 15. *The molecular structure of DNA and representation of the chemical bonds that are formed in PCR.*

Amplification by PCR is characterized by distinct phases (see Figure 16b).

I. **The exponential phase**. The amount of DNA is doubled after each cycle.
II. **The linear phase**. Starting materials, primers and dNTP are consumed and the amplification rate decelerates.
III. **The plateau phase**. The reaction ceases due to depletion of the starting materials and the products may degrade.

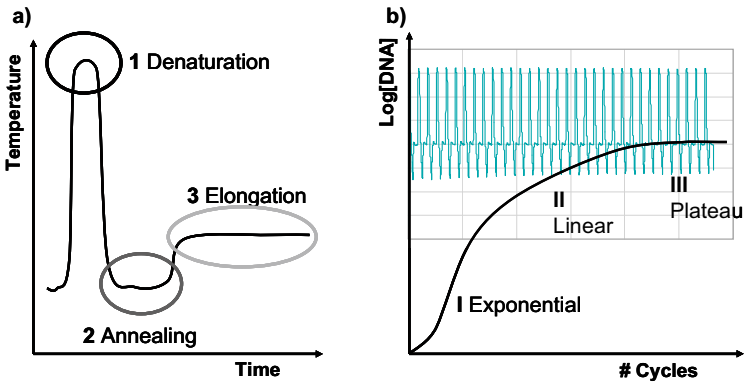


Figure 16. a) *A graph showing one PCR cycle.* b) *The three phases of a PCR.*

1.6.1 Reaction Parameters

Many variables must be considered when setting up a PCR experiment. The most important are discussed below.[226]

- **The DNA template**. The nature of the DNA fragments can differ considerably, being easy or difficult to amplify. For example, as guanine (G) and cytosine (C) interact with three hydrogen bonds in double-stranded DNA, G–C-rich sequences tend to be more difficult to melt than regions with a high content of adenine (A) and thymine (T). Considerably more of the original DNA is needed when amplifying genetic DNA than plasmid or phage DNA. However, high amounts can lead to concomitant amplification of nonspecific PCR products.
- **DNA Polymerases**. DNA polymerases extend the new DNA molecule in the 5′–3′ direction. Taq Pol is the most commonly used DNA polymerase. Its optimum activity is in the temperature range 75–85 °C, and it survives for several minutes at 97.5 °C.[228] Taq Pol replicates a 1 000-bp DNA strand within 10 s,[228] but its error rate is ~1 in 9000 bp, due to lack of 3′–5′ exonuclease proof-reading activity.[229] Several other DNA polymerases, with different temperature sensitivity, error rate and limit in amplicon length, have been isolated.
- **Primers**. DNA polymerases can only elongate the lagging strand of a double-stranded DNA molecule. Therefore, primers that can anneal to the ends of the template region are needed. Normally, two different primers are added one complementary to the 3′-end and the other to the 5′-end of the DNA template. Longer primers provide higher specificity, but have an increased risk of being self-complementary. The optimal G–C content is 50%, uniformly distributed over the oligonucleotides.
- **dNTPs**. The concentrations of each dNTP should identical, as an imbalance dramatically increases the rate of erroneous incorporation.
- **Buffer**. The reaction should be conducted in a buffer optimal for the chosen DNA polymerase. Buffers based on KCl, Tris-HCl and $(NH_4)_2SO_4$ are most common.
- **Salts**. Different inorganic salts, e.g. $MgCl_2$, modify the properties of the dNTPs, primers and the DNA template. Mg^{2+} ions form complexes with single-stranded DNA, hence stabilizing the molecule and preventing hairpin formations and other artefacts of disadvantageous annealing.
- **Other additives**. With the purpose of enhancing the scope and yield of PCR, a large number of additives have been used, such as trehalose and albumin, which can further stabilize elongated, single-stranded DNA.

1.6.2 Monitoring Techniques

Several different techniques can be used to evaluate the outcome of a PCR. The most important are gel electrophoresis, capillary electrophoresis and real-time PCR.[226]

- **Gel electrophoresis**. Gel electrophoreses (GE) is a size-discrimination technique. The short DNA molecules are less entangled in the matrix and migrate through the gel faster than larger DNA molecules when an electric field is applied. A staining agent, such as ethidium bromide, intercalates between the two strands in the DNA molecules and their positions can be determined under UV illumination. The popularity of this technique is due to the low initial investment cost and the possibility of evaluating several experiments in parallel. The main disadvantages are the poor precision, low sensitivity, poor resolution and the toxicity of the staining agents. The light intensity of the bands can be measured in order to obtain quantitative results, but GE is nowadays regarded as a qualitative technique.
- **Capillary electrophoresis**. Capillary electrophoresis (CE) is based on the same principle as GE, but higher voltages, reduced diffusion and more advanced detection techniques are employed, resulting in better separation and much improved quantification.
- **Real-Time PCR**. The newest, and today the most reliable, method for quantifying the number of amplicons in a sample is real-time PCR (rt-PCR). The methodology involves amplification of the DNA segment by normal PCR, but includes an additive, such as SYBR green or reporter probes, which emit fluorescent light when they bind to DNA. Fluorescence can be detected once the DNA–additive complex has reached a certain concentration. The critical concentration is attained after a certain number of thermocycles, called the cycle threshold, C_T. A more dilute sample will cross the detection threshold after a larger number of thermocycles, resulting in a higher value of C_T.

The outcome of a PCR experiment is often referred to as the amplification efficiency, defined as the average yield of one thermocycle. The slightest change in amplification efficiency will thus lead to a considerable change in the overall yield. In a 33-cycle PCR, an amplification efficiency of 95% will result in an overall yield of 18%, while an amplification efficiency of 85% will furnish less than 0.5% of the product, totally.

The PCR technique is constantly being developed and improved. In 2003, Fermer et al. reported the first MW-enhanced PCR procedure, in which the denaturation and elongation steps were accelerated by dielectric heating.[80]

2 Aims of this Work

The overall aim of these studies was to investigate whether bond formations mediated by MW heating could be applied to various areas of drug discovery. The specific objectives are presented below.

- To design and synthesize inhibitors of malaria proteases in an ongoing medicinal chemistry project.
  These inhibitors should:
  - be potent against PM4 orthologues present in all species that infect man,
  - be devoid of activity against selected human aspartic proteases,
  - be drug-like with a peptidomimetic character,
  - comprise a tertiary alcohol in the transition-state mimicking scaffold.
- To establish novel, MW-assisted synthetic routes to small heterocycles.
  These reaction protocols should:
  - be conducted in one reaction vessel, i.e. be one-pot reactions,
  - proceed without the addition of reagent(s) during the course of the reaction, i.e. be a single-operation reaction,
  - be devoid of strong acids, bases, reducing or oxidizing additives,
  - furnish α,β-unsaturated lactones that can be used as reactants in an ongoing medicinal chemistry project and
  - furnish five- and six-membered lactams, useful as scaffolds in an ongoing medicinal chemistry project.
- To establish a procedure for a MW-heated biochemical reaction, more specifically the polymerase chain reaction.
  The reaction protocol should:
  - be fully automated, i.e. all steps of the thermocycle should be conducted by the MW instrument,
  - have an efficiency comparable to that of conventionally heated PCR and
  - allow preparation on a mL scale.

3 Design and Synthesis of Malarial Aspartic Inhibitors (Paper I)

3.1 Design of Potential Inhibitors of Plasmepsin 4

As discussed in Section 1.3.8, few compounds are efficacious inhibitors of all the plasmepsins found in the DV of Pf. The norstatine-based inhibitor **KNI-10006** is an adaptive inhibitor of several aspartic proteases and with good activity against PfPM4 (see Figure 11).[185,190] A combination of the structural features affording **KNI-10006** its high antiplasmepsin potency and the tertiary-alcohol-based TS mimic that lead to good membrane permeability of the HIV-1 PR inhibitor **Mzs27355**[196] (Figure 11) was hypothesized to result in compounds with good membrane permeability and inhibitory effects on all four PM4s causing malaria in humans (Figure 17). This scaffold-hopping operation generated structures with the P1-phenyl group linked to the α-carbon, in contrast to the β-carbon as in **KNI-10006**.

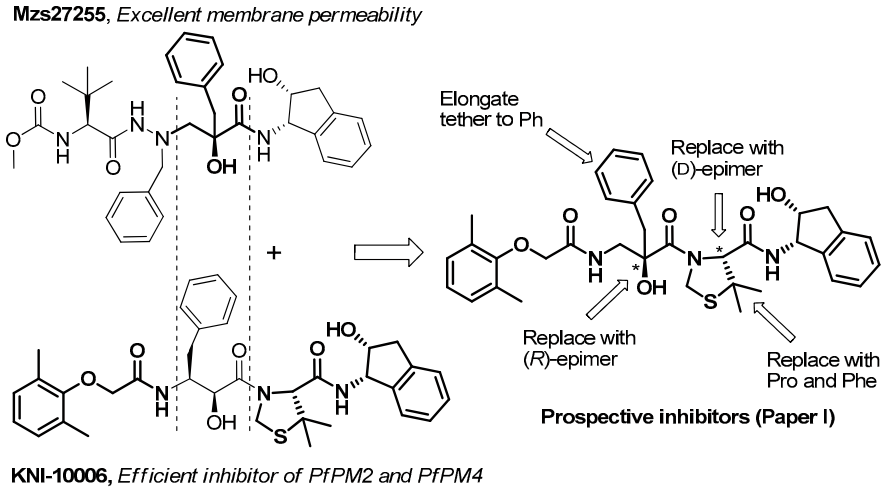


Figure 17. *Design strategy of potential* α*-substituted norstatine-type plasmepsin 4 inhibitors, based on the transition-state-mimicking scaffold (in bold) of* ***Mzs27255*** *and the substituents (in bold) of* ***KNI-10006****.*

With the aim of establishing a SAR, modifications of the stereochemistry of the two central quaternary carbons (marked with * in Figure 17) and the length of the tether to the phenyl group in P1 were made. In **KNI-10006**, two

carbon atoms link the phenyl moiety to the α-carbon in P1. Upon shifting the attachment point to the α-carbon, the distance is reduced by one carbon. It was thus hypothesized that a two-carbon tether to the phenyl would render the flexibility needed for the residue to enter the same lipophilic enzyme-binding pocket as the lead compound **KNI-10006**. A total of eight analogues of **KNI-10006**, four α-phenylnorstatines (R^1 = Ph) and four α-benzylnorstatines (R^1 = Bn), were synthesized according to the retrosynthetic route outlined in Figure 18.

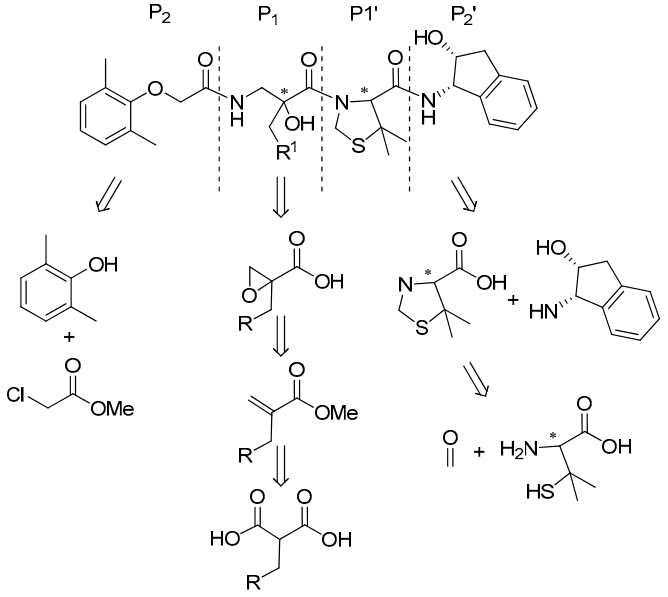


Figure 18. *Retrosynthetic analysis of **KNI-10006*** analogues (Paper I).

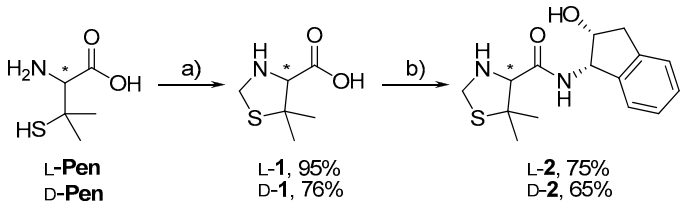
The potential inhibitors were assembled from P2′ to P2 (i.e. from right to left in Figure 18), in order to reduce the use of protective groups and the risk of oxidation of the sulphur in the thiazolidine ring.

Four additional structures encompassing an L-proline (L-Pro) or an L-phenylalanine (L-Phe) residue in P1′ were synthesized for three reasons: 1) the natural substrates of the plasmepsins all have Phe in P1′, while HIV-1 protease cleaves substrates with Pro in P1′ (Table 4, p.33).[185] Hence, the incorporation of L-Phe, could lead to selective inhibitors and L-Pro to adaptive inhibitors; 2) to confirm the SARs observed by Li et al.[172] and Beyer et al.,[186] and 3) a simplified synthetic route, similar to the one yielding **Msz2755**,[196,230] could be employed if analogues were to be realized.

3.2 Synthesis of Potential Plasmepsin Inhibitors

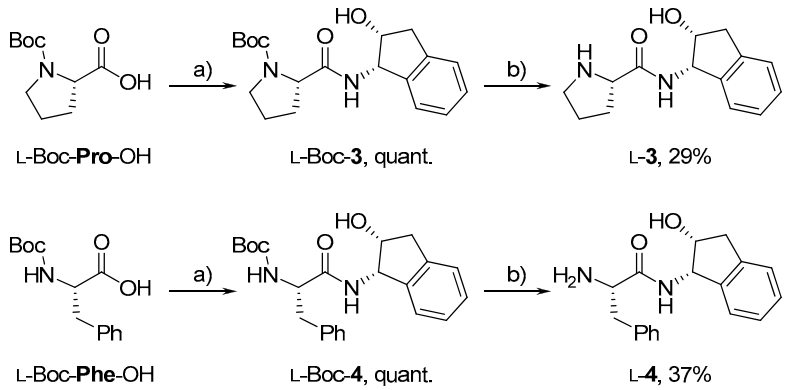
The P1′ residue of **KNI-10006** and the prospective analogues originate from a non-naturally occurring thioproline derivative, (*R*)-5,5-dimethyl-thiazolidine (L-Dmt) carboxylic acid, which was prepared by cyclocondensation from (*R*)-penicillamine (L-Pen) and formaldehyde. The reaction was sluggish (> 15 h) and poor-yielding (53%) under conventional conditions,[231] hence a MW-assisted reaction protocol was developed. The sample was irradiated with MWs for 5 min and cooled in cold water or in the fridge, which resulted in subsequent precipitation of the product L-**1** in a much improved yield (95%) (Scheme 4). The P1′P2′ moiety, L-**2**, was obtained in a good yield (76%) by an amidation reaction between L-**1** and *cis*-(1*S*,2*R*)-1-amino-2-indanol using EDC and HOBt as coupling reagents. A similar procedure reported by Kiso et al. includes two additional steps: the *N*-protection of L-**1** with a *tert*-butyloxycarbonyl group (Boc), and deprotection prior to obtaining L-**2**.[191] This proved to be unnecessary due to the significant difference in reactivity of the primary indolamine and the secondary amine in the thiazolidine ring. Indeed, only small amounts of homocoupled **1** were detected by LC-MS in the reaction mixture.

The other epimer, D-**2**, was prepared in an analogous manner, but gave slightly lower yields, 76% of D-**1** and 65% of D-**2** (Scheme 4).



Scheme 4. *Microwave-assisted synthesis of the P1′P2′ structures encompassing Dmt. Reagents: a) HCHO (aq), pyridine, 110 °C (MW), 5 min, b) (1S,2R)-1-amino-2-indanol, EDC, HOBt, CH_2Cl_2, RT, 1 h.*

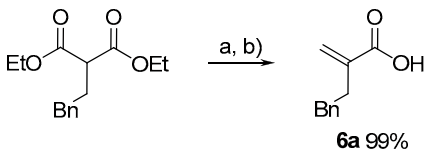
The preparation of the prime-side motifs comprising natural amino acid residues, L-**3** and L-**4**, was conducted in a similar way from Boc-protected L-Pro and L-Phe, respectively (Scheme 5). The amide coupling reaction proceeded to full conversion, and Boc-L-**3** and Boc-L-**4** were isolated in quantitative yields. However, problems in the extraction of the amphiphilic free amines, L-**3** and L-**4**, severely affected the outcome of the deprotection (Scheme 5).



Scheme 5. *Synthesis of P1′P2′ structures encompassing proline and phenylalanine. Reagents: a) (1S,2R)-1-amino-2-indanol, HOBt, EDC, CH_2Cl_2, RT, 1 h; b) HCl (4 M, 1,4-dioxane), RT, 5 h.*

The tertiary alcohol in the TS-mimic is derived from epoxy acids (**5a**,**b**). Ekegren et al., accomplished the synthesis of enantiomerically pure **5b** in three steps from benzyl acrylic acid (**6b**), via a lactic acid ester derivative, in 58% over-all yield.[232] However, the application of this strategy failed to furnish the phenethyl analogue **5a**. Efforts were made to develop a fast, high-yielding alternative procedure affording racemic **5a**,**b**, to be separated by chiral chromatography or as an epimer after coupling with the diastereomeric prime side.

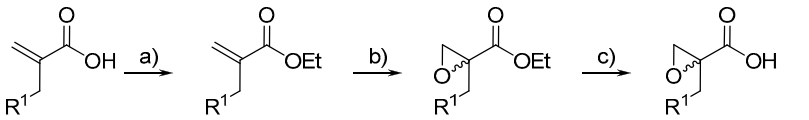
In analogy with the reported synthesis of **6b**,[233] **6a** was obtained by a Knoevenagel reaction from 2-phenethylmalonic acid, which was prepared by hydrolysis of the corresponding ethyl ester (Scheme 6).



Scheme 6. *Preparation of phenethyl acrylic acid. Reagents: a) NaOH, H_2O/THF/MeOH (1/2/1.2), reflux, 30 min; b) HCHO (aq), $HNEt_2$, RT, 30 min then reflux, 3 h.*

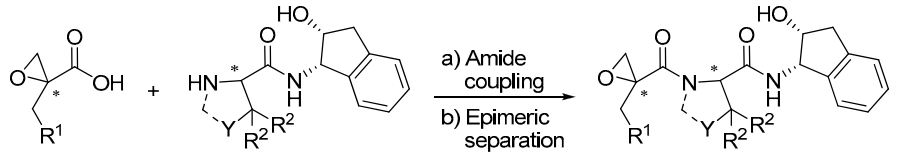
Some initial attempts to perform epoxidation directly on the acrylic acids **6a**,**b** with 3-chloroperbenzoic acid (*m*CPBA), Na_2WO_4/H_2O_2[234] and $NaHCO_3/H_2O_2$[235] were not successful. Indeed, methylacrylic acid is the single terminal olefin with two substituents, one of which is a carboxylic acid, which is known to form epoxides in less than three synthetic steps.[234-236] As ethyl esters of **6a**,**b** have been reported to be epoxidized by *m*CPBA,[237] a fast MW-assisted protocol for direct esterification was developed and applied to the synthesis of **7a**,**b** (Scheme 7). The acrylic esters were oxidized to epoxy esters **8a**,**b** in good yields (81% and 87%, respectively), which were then

hydrolysed in ethanolic KOH, providing the key epoxy acids **5a**,**b** in 67% and 71% yield, respectively, over three steps (Scheme 7). Slightly lower yields were consistently obtained in the transformations of the phenethyl compounds **5**–**8b** (R^1 = Bn).



Scheme 7. *Synthesis of the P1 moieties. Reagents: a) $SOCl_2$, EtOH, 80 °C (MW), 5 min; b) mCPBA, CH_2Cl_2, 18 h; c) KOH (EtOH), RT, 15 min.*

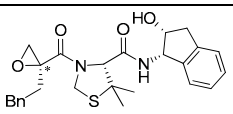
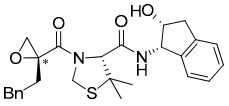
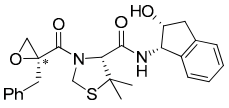
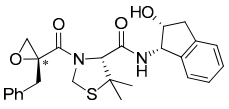
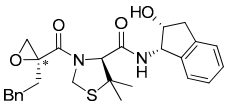
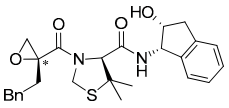
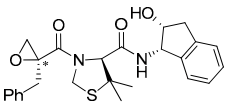
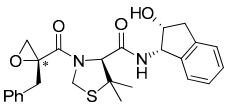
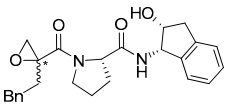
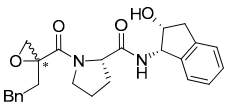
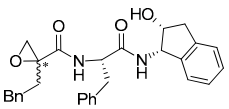
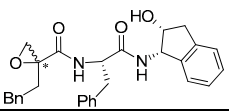
In the subsequent step, the epoxy acids **5a**,**b** were subjected to an amide condensation with the prime-side assemblies (**1**–**4**) (Scheme 8).



Scheme 8. *Amide coupling and subsequent chromatographic separation to obtain the epoxides 9–14 as pure diastereomers.*

Due to the poor nucleophilicity of the nitrogen in the thiazolidine ring of L- and D-**2**, in combination with the steric congestion of the acids **5a**,**b**, normal amide coupling protocols employing EDC/HOBt, HATU and PyBOP did not result in useful amounts of product, if any. The problem was solved by using PyAOP, the aza-analogue of PyPOB, in the synthesis of all epoxides incorporating a cyclic P1′ residue, **9**–**13** (coupling methods I and II, Table 5). The condensation reaction involving the phenylalanine derivative, L-**4**, was performed with PyBOP and *N*,*N*-di-*iso*-propyl-ethylamine (DIEA) (coupling method III, Table 5). The outcome and conditions used for amide coupling and separation are summarized in Table 5.

Table 5. *Synthesis and separation of the epimeric pairs of epoxides **9–14**.*

Com-pound[a]	Structure	P1 *, R^1	P1′	From acid/ amine	Coupling method[b]	Separation method[c]	Yield (%)[d]
(*B*)-**9**		*(S),* Bn	L-Dmt	**5a**/ L-**2**	I	I	24
(*A*)-**9**		*(R),* Bn	L-Dmt	**5a**/ L-**2**	I	I	25
(*A*)-**10**		*(S),* Ph	L-Dmt	**5b**/ L-**2**	II	II	31
(*B*)-**10**		*(R),* Ph	L-Dmt	**5b**/ L-**2**	II	II	45
(*A*)-**11**		*(S),* Bn	D-Dmt	**5a**/ D-**2**	I	I	77
(*B*)-**11**		*(R),* Bn	D-Dmt	**5a**/ D-**2**	I	I	49
(*B*)-**12**		*(S),* Ph	D-Dmt	**5b**/ D-**2**	II	II	50
(*A*)-**12**		*(R),* Ph	D-Dmt	**5b**/ D-**2**	II	II	34
(*A*)-**13**		Bn	L-Pro	**5a**/ L-**3**	I	I	61
(*B*)-**13**		Bn	L-Pro	**5a**/ L-**3**	I	I	62
(*A*)-**14**		Bn	L-Phe	**5a**/ L-**4**	III	I	31
(*B*)-**14**		Bn	L-Phe	**5a**/ L-**4**	III	I	29

a) (*A*) corresponds to the first and (*B*) to the second eluted epimer. b) Coupling methods: I) PyAOP, DIEA, in DCM, RT, 2–5 days; II) As in I, but in DMF; III) PyBOP, DIEA, in CH_2Cl_2, RT 2 days. c) Separation methods: I) column chromatography (silica, EtOAc/*i*Hex) followed by SP-HPLC with a chiral column; II) RP-HPLC with a C8 column. d) Isolated yields, calculated from amine **1**–**4**, assuming that **5** was a racemate.

PyAOP is associated with a pronounced susceptibility to degradation in air. A relationship was observed between the yield and the date when the amide coupling reactions were executed, i.e. in the order **11**, **13**, **9**, **10**, **12**, over a period of 17 months.

As discussed in Section 1.3.8, the stereochemistry of the TS-mimicking hydroxyl group can exert a considerable influence on the inhibitory activity. Thus, the epimeric pairs **9**–**14** were separated by preparative HPLC. For compounds **9**, **11**, **13** and **14** (R^1 = Bn), baseline separation was enabled by a chiral column (Reprosil NR-R) in an HPLC straight-phase (SP) system, using a gradient of *iso*-propanol in *iso*-hexane (separation method I, Table 5). To ensure good epimeric separation, the reaction mixture was pre-purified by flash silica column chromatography. However, this method did not separate the epimers of **10** and **12** (R^1 = Ph). Reversed-phase (RP) HPLC equipped with a conventional C8 column afforded the pure diastereomers (separation method II, Table 5).

The elucidation of the absolute configuration was restricted to epoxides **9**–**12** (P1′ = Dmt). By repeating the synthesis of **10** (L-Dmt) from stereochemically pure (*S*)-**1b**, prepared according to a previously published procedure,[232] and comparing the retention times in two HPLC systems and NMR spectra, the stereochemistry of the first eluted epimer was suggested to be (*S*) and, by elimination, the second to be (*R*). The absolute configurations of the analogues **9** (R^1 = Bn) were deduced by comparing the NMR data and the elution order on RP-HPLC of respective phenylanalogue. The X-ray of the inhibitor derived from the first eluted epoxide **11** (R^1 = Bn, P1′ = D-Dmt) was solved. Hence, the absolute configuration was determined to be (*S*) (Figure 19). A procedure similar to that of the L-Dmt epoxides was used to resolve the stereochemistry of the three remaining D-Dmt analogues.

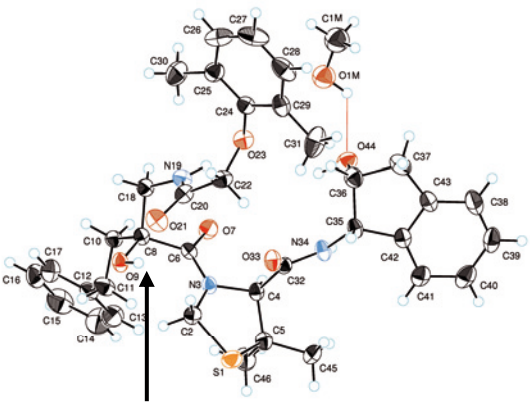
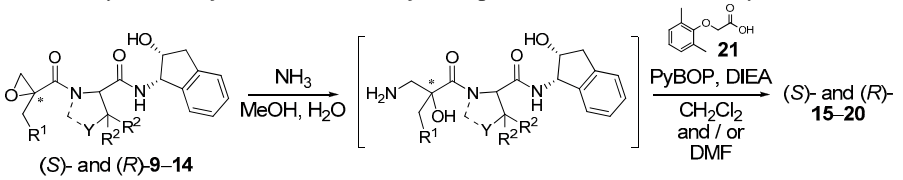
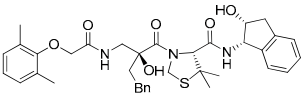
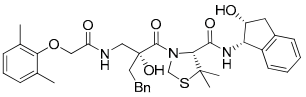
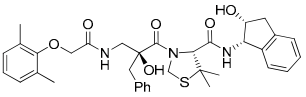
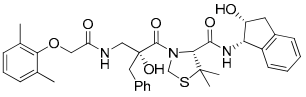
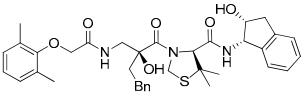
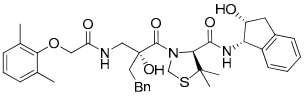
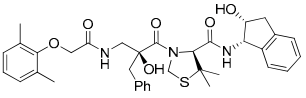
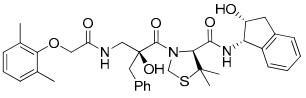
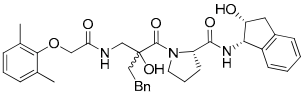
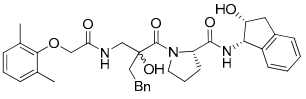
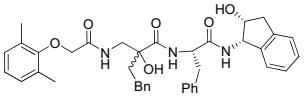
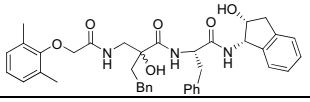


Figure 19. *The crystal structure of inhibitor (*S*)-**17**, enabling the determination of the absolute configuration of the quaternary carbon (C8) of the D-Dmt epoxides.*

The stereopure epoxides were subjected to ammonolysis and the resulting primary amines were condensed with carboxylic acid **21**, prepared using a procedure described in the literature,[238] yielding the final inhibitors (**15**–**20**) (Table 6).

Table 6. *Synthesis of inhibitors **15**–**20** from epoxides **9**–**14** and carboxylic acid **21**.*



Compound	From epoxide	Structure	P1 *, R^1	P1′	Yield[a] (%)
(*S*)-**15**	(*S*)-**9**		*(S),* Bn	L-Dmt	35
(*R*)-**15**	(*R*)-**9**		*(R),* Bn	L-Dmt	57
(*S*)-**16**	(*S*)-**10**		*(S),* Ph	L-Dmt	62
(*R*)-**16**	(*R*)-**10**		(*R*), Ph	L-Dmt	37
(*S*)-**17**	(*S*)-**11**		*(S),* Bn	D-Dmt	55
(*R*)-**17**	(*R*)-**11**		*(R),* Bn	D-Dmt	67
(*S*)-**18**	(*S*)-**12**		*(S),* Ph	D-Dmt	52
(*R*)-**18**	(*R*)-**12**		*(R),* Ph	D-Dmt	57
(*A*)-**19**	(*A*)-**13**		Bn	L-Pro	47
(*B*)-**19**	(*B*)-**13**		Bn	L-Pro	4
(*A*)-**20**	(*A*)-**14**		Bn	L-Phe	53
(*B*)-**20**	(*B*)-**14**		Bn	L-Phe	49

a) Isolated yield.

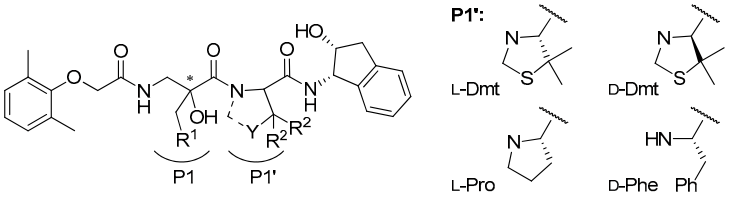
The amide coupling reaction was performed with PyBOP and DIEA in dichloromethane or DMF. The latter solvent was added in order to enhance the solubility of the starting material, but its presence did not seem to change the outcome. The yield was, however, dependent on the result of the HPLC purification. Target compound (*B*)-**19**, for example, was largely overlapped by one of the side products deriving from PyBOP, thus only 4% of pure product was isolated. A temperature-dependent cis/trans-isomerism was observed in the NMR spectra of the compounds incorporating a cyclic P1′ residue (**9**–**13**, **15**–**19**).

3.3 Biological and Computational Evaluation

3.3.1 Biological Results

The synthesized structures were evaluated regarding their ability to inhibit five plasmepsins: PfPM2, PfPM4, PmPM4, PoPM4 and PvPM4, as dissociation constants (K_i) for the inhibitor-enzyme complexes (Table 7).

Table 7. *Plasmepsin inhibition constants of the synthesized* α*-substituted norstatines (the best value on each enzyme is given in boldface).*



Compound	P1: *, R^1	P1′	PfPM2	PfPM4	PmPM4	PoPM4	PvPM4
					K_i (μM)[a]		
(*S*)-**15**	*(S),* Bn	L-Dmt	1.4	3.1	7.9	4.5	0.37
(*R*)-**15**	*(R),* Bn	L-Dmt	7.4	5.5	> 20	6.5	2.2
(*S*)-**16**	*(S),* Ph	L-Dmt	> 20	**0.70**	0.26	**0.11**	0.16
(*R*)-**16**	*(R),* Ph	L-Dmt	> 20	**0.72**	0.25	0.53	0.19
(*S*)-**17**	*(S),* Bn	D-Dmt	**0.44**	6.3	1.1	1.0	0.39
(*R*)-**17**	*(R),* Bn	D-Dmt	7.6	10.7	1.6	0.88	0.34
(*S*)-**18**	*(S),* Ph	D-Dmt	0.83	1.7	**0.21**	1.3	0.16
(*R*)-**18**	*(R),* Ph	D-Dmt	1.7	1.7	0.42	0.61	**0.12**
(*A*)-**19**	Bn	L-Pro	>20	30.2	14.5	7.2	2.2
(*B*)-**19**	Bn	L-Pro	>20	26.9	13.0	10.3	1.3
(*A*)-**20**	Bn	L-Phe	n.d.	>20	n.d.	n.d.	n.d.
(*B*)-**20**	Bn	L-Phe	n.d.	>20	n.d.	n.d.	n.d.

n.d., not determined; a) Determined from the mean of at least three experiments.

The major trends deduced from the data from the assays for each plasmepsin are summarized in Table 8.

Table 8. *Generalization of the relationships between inhibitory activity and the structural features of inhibitors **15**–**20**.*

Inhibitor structural feature			PfPM2	PfPM4	PmPM4	PoPM4	PvPM4
P1	R^1	Bn	+	–	–	–	–
		Ph	–	+	+	+	+
	*	(*S*)	+	=	(+)	+, if P1′= L –, if P1′= D	=
		(*R*)	– –	=	(–)	–, if P1′= L +, if P1′= D	=
P1′	Dmt	L	–	+	(–)	–, if R^1= Bn +, if R^1= Ph	=
		D	+	–	(+)	+, if R^1= Bn –, if R^1= Ph	=
	Pro		– –	– –	–	–	– –
	Phe		n.d.	– –	n.d.	n.d.	n.d.

Explanation of symbols: + more active; – less active; – – much less active; = equally active; (+) slightly, but not significantly, more active; (–) slightly, but not significantly, less active than the alternative structural feature.

No correlation was found between the inhibition of PfPM2 and the orthologues of PM4. This is in general accordance with the previously reported dataset[186] and inhibition data of PfPM2 and PfPM4 inhibition activities.[107] The *α*-substituted norstatines were in general more potent towards the non-*falciparum* plasmepsins. Other, more specific, observations, together with comparisons to the *β*-phenylnorstatines **KNI-10006** and **KNI-764** (Figure 11), are presented below.

PfPM2: The lead compound **KNI-10006** is a very potent inhibitor of PfPM2 (K_i = 0.5 ± 1 nM).[185] The scaffold-hopping operation, furnishing (*S*)- and (*R*)-**15**,**16** as the most analogous compounds, led to a decrease in activity of almost 3000 up to 40000 times. The only structural difference was the transfer of the aromatic residue from the *β*-position to the *α*-position in P1. Interestingly, modification of the P1′, provided the best inhibitors in the series, i.e. the D-Dmt analogues (*S*)-**17** (K_i = 440 nM) and (*S*)-**18** (K_i = 830 nM).

PfPM4: In analogy with PfPM2, **KNI-10006** proved to be a much superior inhibitor (IC_{50} = 15 nM)[190] than the new inhibitors. The two *α*-phenylnorstatines (*S*)-**16** and (*R*)-**16** (R^1 = Ph, P1' = L-Dmt) were the only compounds with K_i values in the nanomolar range (700 nM and 720 nM, respectively).

PmPM4: For comparison, **KNI-764** has a K_i value of 110 nM,[169] being only twice as efficacious as three of the *α*-phenylnorstatines (*S*)-**16**, (*R*)-**16** and (*S*)-**18** (K_i = 210–260 nM). The (*S*) isomer of all epimeric pairs was slightly more potent than the (*R*) isomer, except for (*S*)- and (*R*)-**16**, which were

equally active. D-Dmt as P1′ residue seemed to reduce the difference in potency between the α-phenylnorstatines (R^1 = Ph) and the α-benzylnorstatines (R^1 = Ph).

PoPM4: There are no published data on the inhibition of PoPM4 by comparable small peptidomimetic compounds. (*S*)-**16** was found to be the most potent inhibitor with the best K_i value in the entire dataset (110 nM). The influence of both the length of the tether to the benzene ring and the absolute configuration in P1, were of subordinate importance when D-Dmt was incorporated in P1′ ((*S*)- and (*R*)-**17**,**18**). Another general trend was that the hydroxyl in P1 and the residue in P1′ should point in the same direction, as exemplified by (*S*)-**16** (P1 = (*S*) and Ph, P1′ = L-Dmt) and (*R*)-**18** (P1 = (*R*) and Ph, P1′ = D-Dmt), which are both nanomolar inhibitors with opposite absolute configuration at both stereocentres.

PvPM4: No norstatine structure has previously been tested regarding its inhibitory effect on PvPM4. The K_i values obtained from the PvPM4 assay exhibit less variation than the data from the other iso-enzymes. Out of the ten inhibitors tested, seven were active below 400 nM. All α-phenylnorstatines, (*S*)- and (*R*)-**16**,**18**, (R^1 = Ph) were equipotent (K_i values = 120–190 nM), while the α-benzylnorstatines, (*S*)-**15**, (*S*)-**17** and (*R*)-**17**, (R^1 = Bn) were slightly less active (K_i values = 340–390 nM). (*S*)-**17** was found to be an outlier of the Dmt-encompassing compounds and exhibited considerably less inhibitory activity in the same range as the L-Pro-based compounds (*A*)- and (*B*)-**19** (K_i value = 1300–2200 nM).

The most potent inhibitor in the series of the four PM4 orthologues was the α-phenylnorstatine (*S*)-**16** (K_i value = 110–700 nM), and the most adaptive inhibitors of all five plasmepsin isoenzymes were (*S*)- and (*R*)-**18**.

To the best of the author's knowledge, the P1′ residue D-Dmt has not previously been incorporated into a published aspartic protease inhibitor, and it provided several K_i values in the nanomolar range in all the plasmepsin assays except that involving PfPM4. The best inhibitors of PfPM2 and PvPM4 were based on D-Dmt. The general trend for the α-benzylnorstatines (R^1 = Bn) was that stronger inhibition was observed by the compounds encompassing D-Dmt, whereas the α-phenylnorstatines (R^1 = Ph) were more active when the P1′ residue was an L-Dmt moiety.

The selectivity of all compounds, (*S*)- and (*R*)-**15**–**20**, except for (*B*)-**19**, was tested using an hCatD assay. Compounds (*S*)- and (*R*)-**16**, **18** & **20** were also tested with regard to their inhibitory activity on BACE-1 and the retroviral HIV-1 protease. The ability of compounds (*S*)- and (*R*)-**15**, **17** and (*A*)-**19** to reduce the HIV-1 viral load in infected cells was also evaluated. The results from the assays on isolated enzymes are represented as K_i values and those from the cell assay as EC_{50} values, in Table 9.

None of the assessed α-substituted norstatine structures inhibited hCatD within the measurable range (K_i > 10 μM). The scaffold-hopping procedure resulted in a significant improvement in selectivity. **KNI-10006** (K_i = 2 nM)

is ~4 times more active on PfPM2 than hCatD, to compare with >20 000 for inhibitor (*S*)-**17**. The fact that no compound tested on BACE-1 exhibited any inhibitory activity in the assay, confirms the belief that the α-substituted norstatine core might ensure good selectivity over human aspartic proteases.

Table 9. *Inhibition constants of human aspartic proteases and the HIV-1 protease, and inhibitory activities against HIV-1-infected cells*.

Compound	P1: *, R^1	P1′	hCatD	BACE-1	HIV-1	HIV-1
				K_i (μM)		EC_{50} (μM)
(*S*)-**15**	*(S),* Bn	L-Dmt	> 10.0	n.d.	n.d.	**6.4**
(*R*)-**15**	*(R),* Bn	L-Dmt	> 10.0	n.d.	n.d.	> 10.0
(*S*)-**16**	*(S),* Ph	L-Dmt	> 10.0	> 10.0	> 10.0	n.d.
(*R*)-**16**	*(R),* Ph	L-Dmt	> 10.0	> 10.0	> 10.0	n.d.
(*S*)-**17**	*(S),* Bn	D-Dmt	> 10.0	n.d.	n.d.	> 10.0
(*R*)-**17**	*(R),* Bn	D-Dmt	> 10.0	n.d.	n.d.	> 10.0
(*S*)-**18**	*(S),* Ph	D-Dmt	> 10.0	> 10.0	**0.67**	n.d.
(*R*)-**18**	*(R),* Ph	D-Dmt	> 10.0	> 10.0	> 10.0	n.d.
(*A*)-**19**	Bn	L-Pro	> 10.0	n.d.	n.d.	> 10.0
(*B*)-**19**	Bn	L-Pro	n.d.	n.d.	n.d.	n.d.
(*A*)-**20**	Bn	L-Phe	> 10.0	> 10.0	> 10.0	n.d.
(*B*)-**20**	Bn	L-Phe	> 10.0	> 10.0	**1.0**	n.d.

n.d., not determined

Although the phenylnorstatine–L-Dmt core, published by Kiso et al., have provided many efficacious inhibitors of HIV-1,[191,192] only one analogue, (*S*)-**15** (R^1 = Bn), was active in the cell-based assay. However, (*S*)-**18** (R^1 = Ph, P1′ = D-Dmt) exhibited sub-micromolar activity (K_i = 670 nM) in the assay with the isolated HIV-1 protease. The L-Pro-based compound (*A*)-**19** was not active against HIV-1 protease, while (*B*)-**20**, encompassing an L-Phe residue in P1′, proved to be a moderate inhibitor of HIV-1 protease (K_i = 1000 nM).

3.3.2 Computational Results

The difference in activity between the α-phenylnorstatines (R^1 = Ph) and the lead compound **KNI-10006** was quantified using the linear interaction energy (LIE) method, in which the energy difference between the enzyme–ligand (i.e. the inhibitor) complex and the fully solvated ligand is calculated.[239,240] The binding affinities of **KNI-10006** and **KNI-764** in PM4 have been found to be the result of both polar and nonpolar interactions.[241] In contrast, almost only nonpolar contributions account for the observed binding affinities of (*S*)- and (*R*)-**16**,**18** in PfPM4. This can be explained by the loss of flexibility in the TS-mimicking core, which impairs the important interaction with the catalytic aspartic dyad. Thus, considerably lower K_i values were observed.

In order to obtain reliable results from the LIE method it is important to start from well-docked conformations of the enzyme–ligand complex. In this process, the binding modes of (*S*)- and (*R*)-**16**,**18** in PfPM4 (homology model)[241] and PmPM4 (crystal structure)[169] were explored and compared with the binding orientation of **KNI-764** in PmPM4 observed the solved co-crystal (binding mode I in Table 10),[169] and the binding modes simulated by molecular dynamics (MD) of **KNI-10006** in PfPM4 (binding mode I[194,241]and III[193] in Table 10). Table 10 show the results of the energetically most favoured binding modes of each inhibitor.

Table 10. *Proposed binding orientations in the binding sites, Sn, of PM4 orthologues.*

Inhibitor	Binding mode	P2	P1	P1′	P2′
(*S*)-**16**	II	S2′	S1	S1′	S2
(*R*)-**16**	I	S2′	S1′	S1	S2
(*S*)-**18**	II	S2′	S1	S1′	S2
(*R*)-**18**	I	S2′	S1′	S1	S2
KNI-764[a,169]	I	S2′	S1′	S1	S2
KNI-10006[194,241]	I	S2′	S1′	S1	S2
KNI-10006[193]	III	S2′	S1	S1′	S2′

a) Observed binding mode in the co-crystal with PmPM4.

Interestingly, the *α*-phenylnorstatines with (*S*) configuration in the P1 residue were more stable in an alternative configuration where the positions of P1 and P1′ were interchanged (Figure 20a), and represented a new binding mode (II). Conversely, the (*R*) isomers conserved the observed binding mode (I) of **KNI-764** in PmPM4 (Figure 20b).

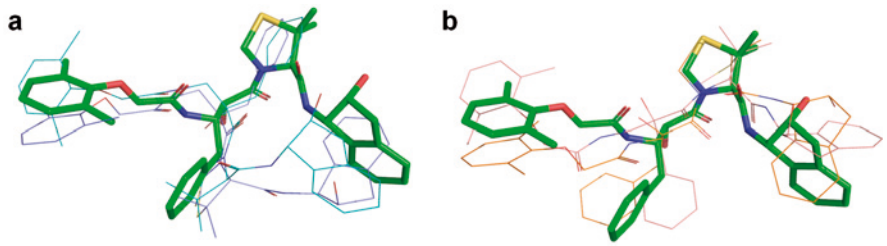


Figure 20. *Comparison of the proposed binding poses of **KNI-10006**[169] (green, binding mode I) and a) (S)-**16** (violet, binding mode II) and (S)-**18** (cyan, binding mode II), and b) (R)-**16** (orange, binding mode I) and (R)-**18** (pink, binding mode I) based on averages from molecular dynamics.*

Binding mode III, which has been proposed for **KNI-10006**,[193] was not stable or provided very unfavourable binding affinity estimates for the four *α*-phenylnorstatine inhibitors.

Figure 21 shows the most frequently occurring hydrogen bond interactions of (*S*)-**16** and (*R*)-**16** in the active site of PfPM4, observed in the extensive MD simulations. Both inhibitors interact with only one of the catalytic aspartic acids, Asp34. Frequent hydrogen bonding with Thr217 was observed for both compounds, but between the P1′ carbonyl of (*S*)-**16** and between the P1 carbonyl of (*R*)-**16**. Wheras the position of the P2 residue was very similar, the P2′ indanolamines showed a flipped orientation with regard to the other epimer. The P1 phenyl residue in (*S*)-**16** interacted with Tyr77 and residue in (*R*)-**16** with Tyr192.

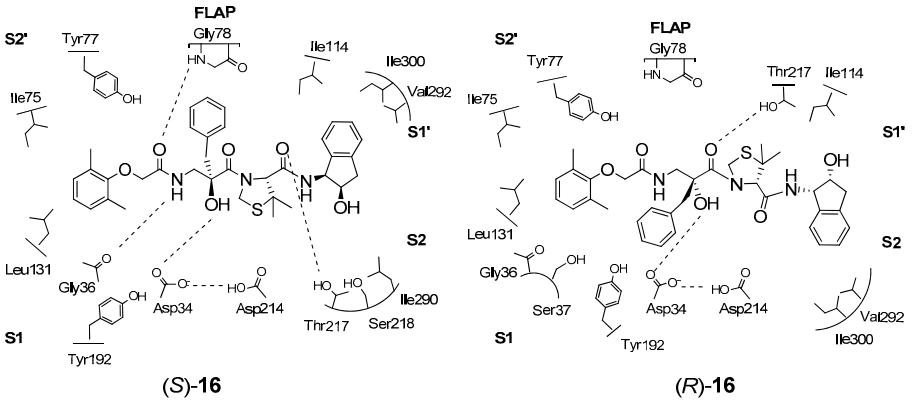


Figure 21. *Schematic illustration (MD sanpshot) of the predicted binding interactions of inhibitors (S)-**16** and (R)-**16** with the PfPM4 active site.*

The four PM4 orthologues account for a high degree of homology of the amino acid sequence, particularly in the substrate binding site. Nonetheless, large variations in potency were observed in a set of inhibitors with, *a priori,* subtle structural modifications. No comparable dataset has been found in the literature and, to the best of the author's knowledge, this investigation of the impact of stereochemical relationships on the affinity to the DV-PM in Pm, Po and Pv is unique. Despite the correlation between the potency on PfPM4 and the other PM4 orthologues, it will probably be necessary to assess all the iso-enzymes to ensure the prevention of haemoglobin degradation, regardless of the species.

4 Development of Lactam and Lactone Formations (Papers II and III)

4.1 Domino Reactions

In order to produce lead compounds that can meet the requirements of a novel drug with the potential of paying back the investments, the chemical structure should be intrinsically complex in a unique fashion, otherwise the intellectual property rights can not be well protected,[242] and the compounds may be promiscuous and cause side effects.[243] However, in order to produce revenue, the synthesis of the compound must be cost effective and scale-up feasible. This is only possible if the structural complexity originates from high-yielding reactions with simple starting materials in an operation-efficient way.[244] One way to accomplish this is to apply domino* reactions.[245]

Tietze's definition of such a reaction is: "a process involving two or more bond-forming transformations (usually C–C bonds) which take place under the same reaction conditions without adding additional reagents and catalysts, and in which the subsequent reactions result as a consequence of the functionality formed in the previous step".[246] The requirement for successfully completed domino reactions is the use of reactive, yet selective, reagents.

4.2 Formation of Cyclic and Non-cyclic *α*,*β*-Unsaturated Amides and Esters (Paper II)

4.2.1 (Triphenylphosphoranylidene)ethenone

(Triphenylphosphoranylidene)ethenone, **22**, also known under the names Bestmann's ylide,** triphenylphosphoranylideneketene or keteneylidenetriphenylphosphorane, is an example of a multipurpose reagent that can be used to introduce a carbon–carbonyl building block into a target structure. The first publication on the synthesis and reactivity of **22** appeared in

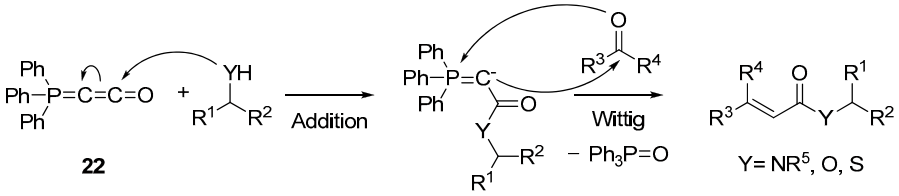
* In paper II, the term cascade reaction is used, but it is ambiguous as the word cascade is applied for many other phenomena in science.

** Not to be confused with Bestmann-Ohira's reagent, dimethyl-1-diazo-2-oxopropylphosphonate.

1966.[247] The reactivity of **22** towards nucleophiles was observed in the very first studies.[247,248] Since then, extensive research on the ability of **22** to undergo nucleophilic attack (on the carbonyl carbon) and electrophilic attack (on the phosphorane carbon) has been conducted by first Bestmann[249,250] and later his coworker Schobert.[251-259] With aldehydes, carboxylic acid esters or ketones with either an adjacent nucleophilic group in α or β-position **22** can form various heterocycles, such as butenolides, tetronic acids, tetronates, coumarins, benzoxepinones and their N- and S-analogues as an alternative to the synthetic routes discussed in Sections 1.4.2 and 1.5.1, respectively.[252] The observed stereochemical conservation of the starting materials is a particularly valuable feature from a medicinal chemical point of view.[260-263]

While not Wittig-active itself, **22** reacts with a host of electrophiles to yield Wittig-type reagents, which can participate in subsequent intra- or intermolecular olefination reactions.[250,261]

The incorporation of a carbonyl-methylene group by **22** is accomplished by a domino reaction triggered by a nucleophilic addition to the carbonyl carbon, followed by a Wittig olefination reaction, as exemplified by the reaction types described in Paper II (Scheme 9). The reaction conditions are in general mild and regioselective, but require long refluxing times (12–48 h).[250,252,264]



Scheme 9. *Domino reactions with (triphenylphosphoranylidene)ethenone. If R^2 and R^3 are connected a cyclization will occur in two steps.*

Despite its intrinsic reactivity, **22** is not degraded in dry air and can be stored at room temperature.[265] Today, it is commercially available, for example, from Sigma-Aldrich and Merck.

4.2.2 Synthesis of α,β-unsaturated Lactones and Lactams

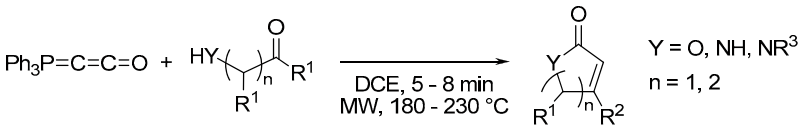
If the nucleophile and the carbonyl illustrated in Scheme 9 are present in the same molecule, unsaturated heterocycles will be formed. In order to reduce the reaction times the published reaction conditions were modified into a general MW-mediated protocol. The reported reactions had been conducted in high-boiling-point solvents such as toluene or xylene, which are not suitable for MAOS as they do not couple with the dielectric field. Consequently, the solvent was replaced by dichloroethane (DCE), which possesses a high loss tangent and similar solvation properties as toluene. The reaction times

were reduced to 5–10 min, while the temperature was increased to 180–230 °C. The acquired reaction conditions were applied to six *o*-functionalized benzylic carbonyl compounds (**23a**–**f**) and five *α*-functionalized aliphatic carbonyl compounds (**23g**–**k**). The reactions were evaluated in terms of product purity calculated as the ratio between the product peak area and the total peak area of the LC-UV chromatogram (254 nm), from which the areas of the side products triphenylphosphine oxide and methyldiphenylphosphine oxide had been subtracted. To verify the correlation between the purity defined in this way and the actual yield, compound **24j** was isolated. The results are summarized in Table 11.

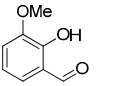
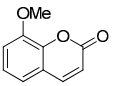
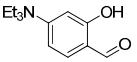
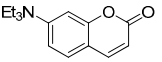
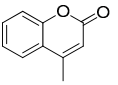
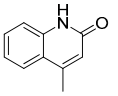
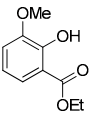
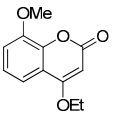
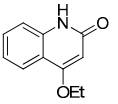
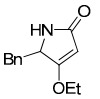
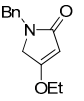
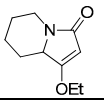
The two *o*-hydroxy benzaldehydes, **23a**,**b**, reacted well with **23** to yield coumarins **24a**,**b** at high purity (97% and 84%). The conversion of *o*-hydroxyacetophenone **23c** and *o*-aminoacetophenone **23d** at 180 °C was unsatisfactory, but increasing the temperature to 230 °C furnished 4-methylcoumarin (**24c**) in good purity (63%) and 4-methyl-2-quinolone (**24d**) in acceptable purity (46%). Generally, the esters were less prone to undergo domino reactions and yield heterocycles. By prolonging the reaction time by three min coumarin **24e** and pyrrolinone **24i** were formed in good purities from the esters **23e** and **23i**, respectively (both 88%). The conversion of phenylalanine ethyl ester (**23h**) was enhanced by increasing the temperature to 230 °C, resulting in 73% purity. The conversion of hydroxy ester **23g** was very satisfactory, but the area of the butenolide **24g** peak was very small in the LC-UV spectrum (14%). The product had a low UV absorbance at 254 nm, hence it was hypothesized that the purity did not reflect the actual yield. Accordingly, **24g** was isolated by preparative HPLC which resulted in a good yield of 72%. Amino acid alkyl ester hydrochlorides had to be deprotonated in order to achieve good conversion. In the preparation of pyrrolinone, **24h**, substrate **23h** was furnished by basic extraction of the corresponding hydrochloric salt. In a more convenient procedure, used in the reaction with **23i**, a Lewis base, triethylamine, was added to the reaction mixture prior to MW irradiation, which was prolonged to eight min to yield pyrrolinone **24i** in 88% purity. The additional steric hindrance of the secondary *α*-amino esters **23i**–**k** did not seem to affect the outcome of the reactions. The *N*-alkylated pyrrolinones **24j**,**k** showed good purities (76% and 69%, respectively) and lactam **24j** was isolated in 70% yield by preparative HPLC.

In general, the reactions were pure. Apart from the anticipated product, unreacted substrate and triphenylphosphine oxide, methyldiphenylphosphine oxide was also found in the reaction mixture. It was also produced in the absence of any other substrate and was therefore isolated to verify the structure. The recorded ^{1}H NMR and ^{13}C NMR corresponded to published data.[266,267]

Table 11. *Microwave-accelerated synthesis of functionalized, α,β-unsaturated lactones and lactams promoted by Ph_3PCCO.*



22 **23a-k** **24a-k**

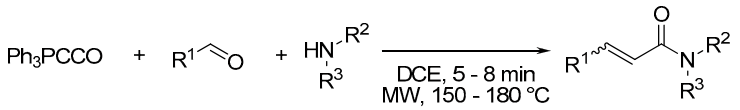
Compound	Starting material	Time (min)	Temp. (°C)	Structure	Yield (%)[a] [Purity (%)][b]
24a	**23a** 	5	180		[97]
24b	**23b** 	5	180		[84]
24c	**23c** 	5	230		[63]
24d	**23d** 	5	230		[46]
24e	**23e** 	8	180		[88]
24f	**23f** 	5	180		[27]
24g	**23g** 	5	180		72 [14]
24h	**23h** 	5	230		[71]
24i	**23i** 	8	180		[88][c]
24j	**23j** 	5	180		70 [76]
24k	**23k** 	5	180		[69]

Reaction conditions: 0.25 mmol **23** and 0.375 mmol **22** in 2.5 mL DCE, in a 2–5 mL sealed tube. a) Products **24g,j** were isolated by HPLC. b) The reactions were evaluated in terms of LC-UV purity (254 nm) given in brackets. c) Et_3N was added to the reaction mixture prior to MW irradiation.

4.2.3 Three-Component Synthesis of *α*,*β*-Unsaturated Amides

The synthon **22** can also be involved in multicomponent reactions, for instance forming *α*,*β*-unsaturated amides from aldehydes and primary or secondary amines.[268] Encouraged by the results presented in Table 11, the same MW-mediated reaction conditions were employed for the synthesis of a small combinatorial library. The generality of the reaction was explored by using five aldehydes (**25a**–**e**), of benzylic, allylic and aliphatic character, and five amines (**26a**–**e**) representing both primary and secondary amines. In order to adhere to the concept of combinatorial chemistry, stock-solutions of the aldehydes **25** and the amines **26** were prepared in DCE. The resulting *α*,*β*-unsaturated amides (**27a**–**u**) were isolated by high-throughput preparative HPLC, and thus the reaction outcome was evaluated by both isolated yield and LC-UV purity (254 nm) (Table 12).

Table 12. *Microwave-accelerated, three-component, domino synthesis of α,β-unsaturated amides promoted by Ph_3PCCO.*



22 **25a-e** **26a-e** **27a-u**

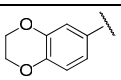
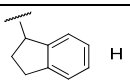
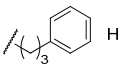
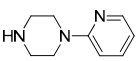
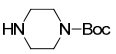
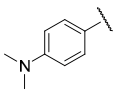
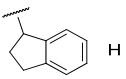
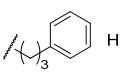
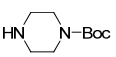
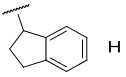
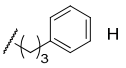
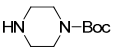
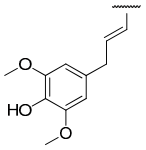
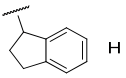
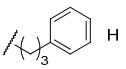
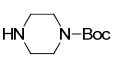
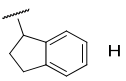
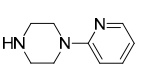
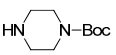
Compound	Aldehyde	R^1	Amine	R^2, R^3	Time (min)	Temp. (°C)	Yield (%)[a] [Purity (%)][b]
27a	**25a**		**26a**		5	180	84 [89]
27b			**26b**		5	180	73 [59]
27c			**26c**		5	180	90 [78]
27d			**26d**		5	180	90 [77]
27e			**26e**		5	150	100 [84]
27f	**25b**		**26a**		5	180	44 [35]
27g			**26b**		5	180	54 [45]
27h			**26c**		10	180	51 [35]
27i			**26e**		5	150	66 [77]

Table 12. *Continued.*

27j	**25c**		**26a**		5	180	81 [76]
27k			**26b**		10	180	74 [83]
27l			**26c**		5	180	88 [90]
27m			**26e**		5	150	74 [91]
27n	**25d**		**26a**		5 10	180	43[c] 85[c]
27o			**26b**		5 10	180	42[c] 66[c]
27p			**26c**		10	180	68 [58]
27q			**26b**		5	150	99 [63]
27r	**25e**		**26a**		5	180	35 [51]
27s			**26c**		5	150	38 [18]
27t			**26d**		10	180	56 [55]
27u			**26e**		5	150	92 [74]

Reaction conditions: 0.125 mmol **25**, 0.15 mmol **26**, and 0.165 mmol **22** in 2 mL of DCE, in a 2–5 mL sealed tube. a) All products **27a**–**u** were isolated by HPLC. b) The reactions were evaluated in terms of LC-UV purity (254 nm), given in brackets. c) No LC-UV purity could be calculated due to the superposition of the triphenylphosphine oxide peak and only isolated yields are reported.

Of all the aldehydes, 1,4-benzodioxan-6-carboxyaldehyde (**25a**) reacted most efficiently with amines **26a**–**e**, and furnished amides (**27a**–**e**) with the highest yields (73–100%). The reactions with picolinaldehyde (**25c**) also resulted in high yields of amides **27j–m** (74–88%). The reactions with 4-dimethylaminobenzaldehyde (**25b**) and cyclohexylcarboxaldehyde (**25e**) were significantly less successful (yields 44–61% and 35–92%). The cinnamaldehyde (**25d**) was only partly converted after 5 min of processing with mediocre yields of amides **27n**,**o** (43% and 42%, respectively). With an additional 5 min of MW irradiation, the yields were greatly improved (85% and 66%).

Of the amines **26a**–**e**, Boc-protected piperazine, **26e**, resulted in the highest yield with all aldehydes, except with picolinaldehyde, furnishing amides **27e**,**i**,**m**,**q**,**u** in yields of 100%, 66%, 74%, 99% and 92%, respectively. However, all the reactions involving **26e** had to be carried out at 150 °C, in order to reduce the risk of heat-induced Boc deprotection.[269]

With few operational steps, many of which were fully automated, 21 α,β-unsaturated amides were synthesized and isolated. Despite serial MW irradiation of the samples, the total reaction heating time was merely 2 h, 15 min. With a few exceptions, good to excellent yields (38–100%) of amides **27** were recovered from the reaction mixtures. The correlation between the isolated yields and LC-UV purities were generally good, although the yields tended to be slightly underestimated. Due to the highly automated procedure, some products were too contaminated with phosphine oxide species to be isolated. This was especially true for the reactions involving amine **26d**, and only two products could be satisfactorily retrieved from the reaction mixture. Only one of the α,β-unsaturated amides **27a**–**u** in Table 12 has been published previously.[270] Unfortunately, an attempt to repeat the automated fashion of generating a library with α,β-unsaturated esters failed, mainly due to problems in the LC-MS detection of the products.

Two types of domino reactions were successfully performed with Bestmann's ylide (**22**) in a MW reactor, providing α,β-unsaturated lactones, lactams and amides within a few minutes, compared to hours or even days under conventional reaction conditions. Other groups have performed MW-accelerated synthesis with ylide **22**[253,256,258,271] and similar ylides,[272] since the publication of Paper II.

4.3 Lactams from Amines and Lactones (Paper III)

4.3.1 Ionic Liquids in Organic Synthesis

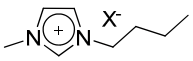
By definition, an ionic liquid (IL) is a salt with a melting point below approximately 100 °C, but is often referred to as a salt which is liquid at room temperature. The low melting point is a consequence of impaired columbic attractions between the ions due to a high degree of asymmetry in the crystal lattice. In most cases this is achieved by combining a relatively large, and often flexible, organic cation with a smaller, inorganic anion.

Although the first ILs were discovered more than a hundred years ago,[273,274] they were considered for several decades to be a scientific curiosity rather than something valuable for industry. However, since the introduction of the first air- and water-stable ILs in the 1990s, the interest for ILs is increasing.[275] Many comprehensive reviews describe their physicochemical properties[276-280] and the applications in organic synthesis.[281-286]

Typical characteristics of ILs are their electrical conductivity and extremely low vapour pressure. Other properties, such as thermal stability, liquid temperature range, solvation properties, miscibility with water and organic solvents, Lewis acidity, Lewis basicity, hygroscopicity, viscosity, etc., have been fine-tuned by altering the cation or replacing the anion.

The most commonly used ILs in organic synthesis today are based on imidazolium ions, particularly 1-butyl-3-methylimidazolium ([bmim]$^+$) with weakly coordinating counterions such as BF_4^- and PF_6^- (corresponding to IL **28a** and **28b**, respectively). Their properties are summarized and compared with those of [bmim]Cl in Table 13.

Table 13. *Physicochemical and solubility properties of two1-butyl-3-methylimidazolium ionic liquids.*[276,277]



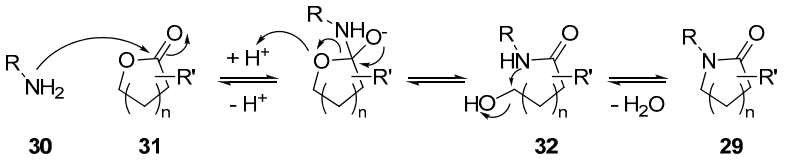
[bmim]X

Property/IL	**[bmim]BF_4 (28a)**	**[bmim]PF_6 (28b)**	**[bmim]Cl**
Melting temp (°C)	–81	10	65
Density at 25 °C (g/mL)	1.208	1.373	-
Viscosity at 25 °C (cP)	219	450	-
Decomposition temp (°C)	403	349	254
Solubility - miscible	H_2O, EtOAc, MeOH, Tol	Acetone, DCM	H_2O
Solubility - immiscible	Acetone, $CHCl_3$, MeOH	H_2O, *n*-HexH, Tol	

MW-assisted heating of reactions involving ILs is particularly interesting as they absorb MW irradiation extremely well due to their polar and ionic nature. Due to their attractive properties such as low vapour pressure, high thermal stability and solubility, [bmim]BF_4 and [bmim]PF_6 have been successfully employed as catalysts, solvents and cosolvents in organic synthesis.[287-289] In addition, ILs have been added as an auxiliary to ensure rapid MW-mediated heating in MW-transparent solvents.[288] Recently, ILs were found to stabilize a catalytic enzyme subjected to MW irradiation.[290]

4.3.2 Lactamization of Lactones

An alternative method of synthesizing saturated lactams (**29**) is the condensation of amines (**30**) and lactones (**31**).[200] The reaction is proposed to proceed by a three-step reversible mechanism, via an acyclic hydroxyamide intermediate (**32**), as outlined in Scheme 10.[291]



Scheme 10. *Proposed mechanism for the formation of lactams from amine and lactones.*

Previously reported methodologies for providing *N*-substituted lactams from lactones usually involve reaction conditions that are unattractive from a medicinal chemical point of view, such as:

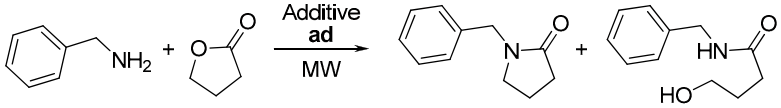
- long reaction times at high temperatures,[292-294]
- strong Brønsted acids as catalysts,[295-298]
- strong Brønsted bases as additives[299,300] and
- multistep transformations, in which the intermediate, i.e. the *ω*-hydroxyamide, is activated by transforming the hydroxyl group into a better leaving group.[301-303]

4.3.3 Identification of Reaction Parameters

The main purpose of developing a MW-assisted protocol for lactam formation was to 1) reduce the reaction time, 2) simplify the reaction procedure, 3) improve product purity, and 4) enlarge the pool of starting material that can withstand the reaction conditions, in order to enable the synthesis of structures in an ongoing medicinal chemistry project.

Inspired by the earliest reported lactamization,[292,293] neat conditions were applied in the initial experiments (entries 1–3, Table 14), using benzylamine (**30a**) and γ-butyrolactone (**31a**) as model substrates. However, in the absence of additives only limited amounts of *N*-benzylbutyrolactam (**29a**) were formed. Instead, the acyclic intermediate *N*-benzyl-*γ*-hydroxybutyroamide (**32a**) was the predominant species in the reaction mixture (entries 1–3, Table 14). MW heating at 150 °C for 15 min did not lead to full conversion of the yield-limiting *γ*-butyrolactone (**31a**). Despite increasing the reaction temperature to 220 °C and extending the reaction time to 35 min, the yield of **29a** remained unsatisfactorily low (entry 2, Table 14). To investigate whether further prolongation of the reaction time would improve the outcome, a sample was irradiated with MW at 220 °C for 7 h. Aliquots were withdrawn through the vial septum and analysed using GC-MS every hour for the first three hours. After MW irradiation of a further 4 h, the products **29a** and **32a** were isolated (entry 3, Table 14). The four GC-MS chromatograms were strikingly similar to that for entry 2 in Table 14, as were the yields.

Table 14. *Optimization of reaction parameters for MW-assisted γ-lactam formation.*



30a **31a** **29a** **32a**

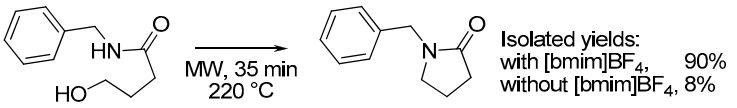
Entry	Additive (**28**)	Cosolvent	Molar ratio (**30:31:28**)	Temp. (°C)	Time (min)	Yield of **29a** (%)	Yield of **32a** (%)
1	-	-	3:1:0	150	15	8	78
2	-	-	3:1:0	220	35	16	47
3	-	-	3:1:0	220	420[a]	17	47
4[b]	AcOH	1,4-Dioxane	2:1:0	180	30	0	n.d.
5[c]	HCl, 4.0 M	1,4-Dioxane	2:1:0	180	30	50% conv.	n.d.
6[c]	PTSA	1,4-Dioxane	3:1:1	180	35	60% conv.	n.d.
7	PTSA	1,4-Dioxane	3:1:1	220	35	89	-
8	[bmim]BF_4	-	3:1:1	180	35	82	-

Reaction conditions: All reactions were performed with 2.0 mmol **31a**, using 0.5-2 mL sealed tubes. All yields, except for entries 5 and 6, denote isolated yields with a purity >95% according to GC-MS. a) 3 × 60 min + 240 min. b) No **29a** could be isolated, and the yield of **32a** was not determined (n.d.). c) Only the specified percentage of **31a** was converted to **29a** and the yield of **32a** was not determined (n.d.).

The investigation proceeded with the addition of various Brønsted acids to the reaction protocol, as this has been reported by several other groups to be a successful methodology.[295-298] When 1 equiv. of acetic acid (AcOH) was added, almost all lactone **31a** was consumed, but only trace amounts of lactam **29a** were formed (entry 4, Table 14). In fact, the concomitant acetylation of benzamine, yielding *N*-benzylacetamide, was the dominating reaction, although some formation of hydroxyamide **32a** also occurred. In the experiment with 1 equiv. of HCl (4 M in 1,4-dioxane), lactam **29a** was detected in the reaction mixture, but unreacted **31a** remained, as well as considerable amounts of uncyclized **32a** (entry 5, Table 14), therefore no products were isolated. At 180 °C, 1 equiv. of *p*-toluenesulphonic acid (PTSA) dissolved in 1,4-dioxane, gave a similar result (entry 6, Table 14). By raising the temperature to 220 °C, successful lactamization was accomplished with a 89% yield of lactam **29a**, full conversion of **31a** and only trace amounts of **32a** (entry 7, Table 14). PTSA, is a very strong Brønsted acid, which is not tolerated by substrates with acid-sensitive functional groups. Inspired by lactamization procedures promoted by polar additives, such as $ZnCl$[304] and zeolites,[305,306] an experiment with the IL [bmim]BF_4 (**28a**) was performed. It proceeded directly in a two-step reaction, showing full conversion of **31a** to **29a** in a satisfactory yield of 82% (entry 8, Table 14).

In an attempt to explain the role of **28a** in the direct, MW-assisted lactamization process, the acyclic hydroxyamide, **32a**, was and used as the substrate in two experiments, one with the addition of **28a** and one in the absence of IL (Scheme 11). MW heating of **32a** without an additive yielded

only 8% lactam, while in the presence of IL, the yield was 90%. These results suggest that the IL promotes cyclization by facilitating the nucleophilic substitution of the hydroxyl group, but is not necessary for lactone opening, which was readily accomplished without additives and at a lower temperature, i.e. 150 °C (see entry 1, Table 14). Thus, it could be postulated that cyclization is the rate determining step, which was in accordance with the results of a study published by Decker et al.[294]



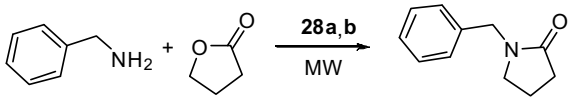
32a **29a**

Scheme 11. *The influence of [bmim]BF_4 on the cyclization of hydroxyamide,* ***32a****.*

The promising result of entry 9 in Table 14 promoted a further investigation of the influence of the reaction parameters, e.g. the molar ratio amine:lactone:IL, the choice of ionic liquid, the reaction temperature, the reaction time, the addition of a cosolvent and the reaction scale. The purpose of using a cosolvent was the prospect of improved stirring and solvation, particularly of very viscous or solid substrates. 1,4-Dioxane was the cosolvent of choice, since it had been identified as an inert and effective cosolvent in entry 7 (Table 14). The consequences of the variations in the reaction conditions can be found in Table 15. The most pronounced trends observed from the investigation of the IL-promoted lactamization can be summarized as follows:

- An excess of amine was essential for successful lactamization (compare entries 1–3 with entry 4 in Table 15). However, a large excess of 5 equiv. resulted in a mere 50% conversion of lactone (entry 5).
- Increasing the amount of IL did not affect the outcome of the reaction (entry 3 in Table 15).
- Replacing [bmim]BF_4 with [bmim]PF_6 had no significant effect on the outcome (compare entries 9 and 12).
- Higher reaction temperatures afforded reaction mixtures with less hydroxyamine, **32a**, and slightly higher yields (compare entry 1 with entry 2 and entry 6 with entry 9).
- Prolonged reaction time resulted in a considerably lower yield (entry 7).
- The addition of 1,4-dioxane and a reduction in the amount of amine resulted in an improved yield (compare entry 9 with entry 10).
- Reducing the reaction scale 10 times to 0.2 mmol did not affect the reaction outcome (compare entry 10 with entry 11).
- Increasing the reaction scale 15 times to 30 mmol did not affect the reaction outcome (compare entry 12 with entry 13).

Table 15. *Optimization of reaction parameters for ionic liquid-mediated γ-lactam formation.*



30a **31a** **29a**

Entry	Ionic Liquid	Molar ratio (**30**:**31**:**28**)	Cosolvent	Temp. (°C)	Time (min)	Reaction vial size[a]	Scale (mmol)	Yield (%)
1	**28a**	2:1:1	-	170	35	XS	1	60
2	**28a**	2:1:1	-	180	35	XS	1	77
3	**28a**	2:1:2	-	180	35	XS	1	78
4	**28a**	1:3:1	-	180	35	XS	1	51
5	**28a**	5:1:1	-	180	35	S	2	n.d.[b]
6	**28a**	3:1:1	-	180	35	S	2	82
7	**28a**	3:1:1	-	180	60	S	2	39
8	**28a**	3:1:1	-	200	35	S	2	70
9	**28a**	**3:1:1**	**-**	**220**	**35**	**S**	**2**	**86**
10	**28a**	**3:2:2**	**1,4-Dioxane**	**220**	**35**	**S**	**2**	**99**
11	**28a**	3:2:2	1,4-Dioxane	220	35	XS	0.2	95
12	**28b**	3:1:1	-	220	35	S	2	93
13	**28b**	3:1:1	-	220	35	L	30	88

All yields denote isolated yields with a purity >95% according to GC-MS. a) L = 10–20 mL; S = 0.5–2 mL; XS = 0.2–0.5 mL sealed tubes. b) n.d., not determined. **29a** was not isolated, as only ~50% of **31a** was consumed according to GC-MS.

Although the yield was slightly higher when employing [bmim]PF_6 instead of [bmim]BF_4, the work-up was considerably easier in the [bmim]BF_4-promoted reaction, due to differences in the solubility properties of these ILs (see Table 13). The use of cosolvent proved to be of great importance for a successful 0.2-mmol reaction. The reactions were typically worked up by extraction with EtOAc and sat. NH_4Cl (aq), thus removing the excess amine and most of the IL. The crude product was reduced *in vacuo*, and the lactam was isolated by flash column chromatography (silica, EtOAc:MeOH or EtOAc:*i*Hex). The product purity was verified to be >95% by GC-MS and NMR. In the case of entry 10, Table 15, the crude product fulfilled this criterion and no chromatography was needed.

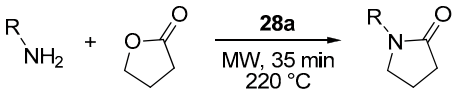
Two procedures were identified as most interesting for the subsequent investigations, both heated in a 0.5–2 mL reaction vial for 35 min at 220 °C.

- Method **A**: Corresponding to entry 9 (Table 15): 6 mmol (3 equiv.) amine, 2 mmol lactone and 2 mmol **28a**.
- Method **B**: Corresponding to entry 10 (Table 15): 3 mmol (1.5 equiv.) of amine, 2 mmol lactone, 2 mmol **28a** and 1 mL 1,4-dioxane as cosolvent.

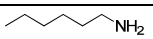
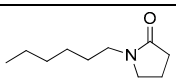
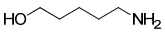
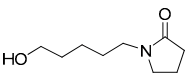
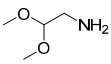
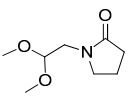
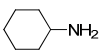
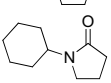
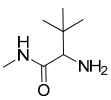
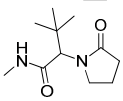
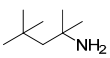
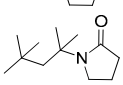
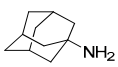
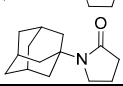
4.3.4 Preparative Results

Different primary amines were reacted with γ-butyrolactone in order to explore the generality of the methodology. All lactamization with aliphatic amines (**30b**–**j**) was conducted according to method **A**, apart from one experiment. The transformations yielding aliphatic lactams are summarized in Table 16.

Table 16. *Lactam formation from γ-butyrolactone and aliphatic amines.*



30b-j **31a** **29b-j**

Entry	Amine	Method	Product	Yield (%)
1	**30b** 	A	**29b** 	96
2	**30c** 	A	**29c** 	92
3	**30d** 	A[a]	**29d** 	54
4	**30e** 	A	**29e** 	93
5	**30f** 	A	**29f** 	85
6	**30g** 	A	**29g** 	38
7		B		62
8	**30h** 	A	**29h** 	30
9	**30i** 	A	**29i** 	23
10	**30j** 	A	**29j** 	73

Reaction conditions: 2 mmol **31a** and 2 mmol **28a** in a 2–5 mL sealed tube. All yields denote isolated yields with a purity >95% according to GC-MS. Method **A**: 6 mmol of **30a**, without a cosolvent. Method **B**: 3 mmol of **30a**, *without* a cosolvent. a) The temperature was reduced to 210 °C after 21 min of processing due to high pressure.

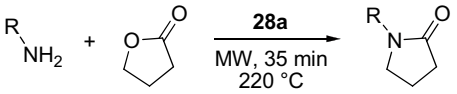
Excellent yields were obtained from the reactions with non-functionalized amines adjacent to a primary or secondary carbon (entries 1, 4 & 5, Table 16). Interestingly, 5-amino-pentanol, **30c**, incorporating two nucleophilic groups, yielded exclusively lactam **30c** (92%), and the possible transesterification did not occur (entry 2, Table 16). An experiment was conducted with

the similar compound, amine 2-amino-ethanol. According to GC-MS, the lactamization process was very successful and went to completion, but the product was too polar and volatile to be isolated by the general procedure. No further experiments could be performed due to lack of time. The dimethyl acetal **30d** in entry 3, Table 16, furnished lactam **29d** in an acceptable yield (54%). The corresponding aldehyde was not detected in the reaction mixture, which is an obvious advantage of this acid-free lactamization protocol. During the course of the reaction, the temperature had to be adjusted to 210 °C to keep the pressure within the limits of the MW instrument. Relatively low yields were obtained with amines encompassing *tert*-butyl groups, such as *tert*-leucine methyl amide (**30g**) *tert*-butylamine (**30h**) and 1,1,3,3-tetramethylbutylamine (**30i**) (entries 6–9, Table 16). Unsubstituted γ-lactam was found as the major side product in entries 8 and 9, presumably due to facile tert-carbocation formation of the amine substituents. Nonetheless, by reducing the excess of the solid amine **30g**, the yield was improved from 38% to 62% (entry 7, Table 16). Another solid amine, **30j**, with a very bulky adamantanyl substituent, afforded lactam in a good yield (73%) under standard method **A** conditions (entry 10, Table 16).

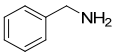
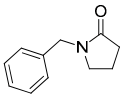
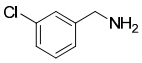
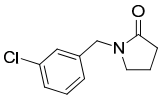
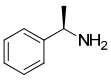
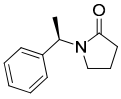
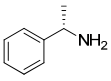
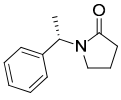
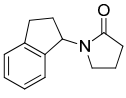
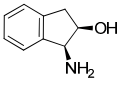
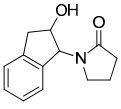
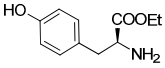
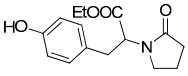
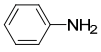
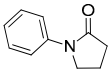
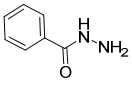
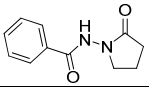
A second group of amines (**30k–q**) and a hydrazide (**30r**), all carrying aromatic substituents, were investigated as condensation partners in the protocols for direct lactamization. The results are presented in Table 17, with the previously described reactions of **30a** and **31a** (entries 9 and 10, Table 15) included for comparison (entries 1 and 2, Table 17).

Generally, the amines devoid of functional groups, **30a**,**k**–**m**, afforded lactams, **29a**,**k**–**m**, in excellent yields (entries 1–5, Table 17), including *meta*-chlorobenzylamine, **30k**. These results are in accordance with the outcome of the reactions with the aliphatic amines (Table 16). The exception was 1-indanamine (**30n**) which furnished lactone **29n** in a moderate yield of 47% (entry 6, Table 17). The stereocentre of the enantiopure amines **30l**,**m** remained intact after lactam formation (entries 4 and 5, Table 17). The enantiomeric excess of **29l**,**m** was analysed by optical rotation and by RP-HPLC using a chiral column (see S46, Supporting Information, Paper III). Lactam **29o** also proved to be optically active, suggesting that the two stereocentres of (1*S*,2*R*)-indanolamine (**30o**) were not affected, but this could not be verified due to lack of reference data.[307]

Table 17. *Lactam formation from γ-butyrolactone and amines with an aromatic functionality.*



30a,k-r **31a** **29a,k-r**

Entry	Amine	Method	Product	Yield (%)
1	**30a** 	A	**29a** 	86
2		B		99
3	**30k** 	A	**29k** 	84
4	**30l** 	A	**29l** 	99
5	**30m** 	A	**29m** 	97
6	**30n** 	A	**29n** 	47
7	**30o** 	A	**29o** 	63
8		B		53
9	**30p** 	A	**29p** 	19
10		B		21
11	**30q** 	A	**29q** 	74
12	**30r** 	A	**29r** 	32

Reaction conditions: 2 mmol **31a** and 2 mmol **28a** in a 2–5 mL sealed tube. All yields denote isolated yields with a purity >95% according to GC-MS. Method **A**: 6 mmol **30a**, without a cosolvent. Method **B**: 3 mmol **30a** and 1 mL 1,4-dioxane.

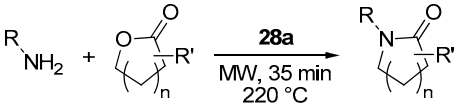
The yield of the reaction was slightly disappointing (63%) and was not improved by employing the general procedure **B** (53%), as described in entries 7 and 8, Table 17. Likewise, the reaction with L-tyrosine ethyl ester (**30p**) gave poor yields (19–21%), regardless of the method used (entries 9 and 10, Table 17). Hydrolysis of the ethyl ester of either **30p** or **30p** was not believed to be the cause of the low yields. Similarly to L-*tert*-leucine methyl amide **30g** (entries 6 and 7, Table 16), amine **30p** is a solid amino-acid derivative. Hence, the work-up and separation of the reaction mixtures of the

highly polar products were difficult. Insufficient magnetic stirring during MW irradiation was believed to be an additional reason for the poor outcome. Obviously, the changes employed in method **B** were not sufficient to compensate for this. Although aniline (**30q**) is a weak nucleophile, *N*-phenyl-*γ*-lactam **29q** was isolated in good yield of 74% (entry 11, Table 17). Rewardingly, also a hydrazide (**30r**) afforded lactam **29r** in an acceptable yield without any alterations to the standard procedure (entry 12, Table 17).

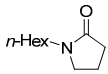
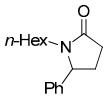
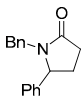
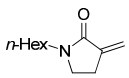
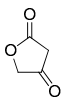
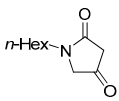
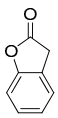
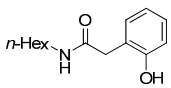
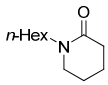
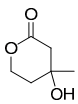
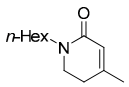
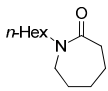
A further investigation of the scope of the reaction was conducted with a set of commercially available lactones, with five, six, or seven ring atoms. All eleven lactones were reacted with *n*-hexylamine (**30b**), and two also with the model substrate benzylamine (**30a**), as indicated in Table 18. The previously discussed reactions of *γ*-butyrolactone **29a** with **30a** (entry 1, Table 16) and **30b** (entries 1 and 2, Table 17) are included in Table 18 as entries 1–3 for comparison.

γ-Phenyl-*γ*-butyrolactone (**31b**) generated lactones **29b** and **29a** in excellent yields with both amines (entries 4 and 5, respectively, Table 18). The steric bulk of the phenyl group in the γ-position did not affect the cyclization process, as the yields are comparable with those of the unsubstituted lactone **31a**. Despite the risk of competing 1,4-attack of amine **30b** on *α*-methylene-*γ*-butyrolactone (**31c**), the corresponding lactam, **29u**, was isolated in a satisfactory yield (entry 6, Table 18). Tetronic acid (**31d**) is also susceptible to numerous side reactions, such as imine formation of the *β*-carbonyl or deprotonation of the *α*-proton, but yielded acceptable amounts of lactone **29v** (entry 6, Table 18). It was thought that a reduction of the excess amine, being basic, would reduce the amount of unwanted transformations (entry 7, Table 18). Indeed, the reaction mixture accounted for fewer components, but the yield was lower, 43% with method **B** and 56% with method **A**. No lactam **29w** was formed from 2-coumaron (**31e**), when either method **A** or **B** was used, because the phenol group of the intermediate **32w** did not undergo substitution. Nonetheless, the amide formation was straightforward and 89% of hydroxyamide **32w** was isolated (entry 9, Table 18). As expected, the results of lactamization proved to be dependent on ring size. The six-atom unfuctionalized *δ*-valerolactone (**31g**) afforded lactams **29x** and **29y** in yields that were good (entries 10 and 11, Table 18), but considerably lower than those of **29b** and **29a** (entries 1–3, Table 18). Mevalonic acid (**31h**), was subjected to concomitant dehydration of the *α*-hydroxyl group. The only lactam product in the reaction mixture was the *α*,*β*-unsaturated lactam **29z**, which was isolated in a yield of 73% after MW heating according to method **A** (entry 12, Table 18). Similarly, in the reaction with *β*-hydroxy-*γ*-butyrolactone, no saturated corresponding lactam could be detected and the reaction mixture was discarded without any further investigation.

Table 18. *Lactam formation from γ-, δ-, and ε-lactones and amines.*



30a,b **31b-h** **29a,b,s-#**

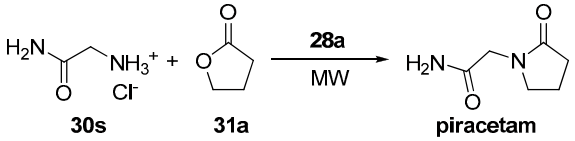
Entry	Amine	Lactone	n	Method	Product	Yield (%)
1	**30b**	**31a** 	1	A	**29b** 	96
2 3	**30a**	**31a** 	1	A B	**29a** 	86 99
4	**30b**	**31b** 	1	A	**29s** 	91
5	**30a**	**31b** 	1	A	**29t** 	93
6	**30b**	**31c** 	1	A	**29u** 	42
7 8	**30b**	**31d** 	1	A B	**29v** 	56 43
9	**30b**	**31e** 	1	A	**32w** 	89[a]
10	**30b**	**31f** 	2	A	**29x** 	65
11	**30a**	**31g** 	2	A	**29y** 	50
12 13	**30b**	**31h** 	2	A B	**29z** 	73[b] 0
14 15	**30b**	**31i** 	3	A B	**29#** 	12 30[c]

Reaction conditions: 2 mmol **31** and 2 mmol **28a** in a 2–5 mL sealed tube. All yields denote isolated yields with a purity >95% according to GC-MS. Method **A**: 6 mmol **30**, without a cosolvent. Method **B**: 3 mmol **30**, with 1 mL 1,4-dioxane as cosolvent. a) No lactam product **29w** was formed. b) No saturated lactam was isolated. c) 240 °C and 4 mL 1,4-dioxane.

Finally, ε-caprolactone, with seven atoms in the ring, was used as a substrate. The corresponding unsubstituted lactam, i.e. ε-caprolactam, is the monomer used for the production of Nylon-6. Not surprisingly, extensive polymerization occurred during processing with **30b**. Using method **A**, only

limited amounts of *N*-hexyl-ε-caprolactam (**29#**) could be isolated (entry 14, Table 18). Thus, method **B** was modified in order to reduce the concentration by adding 4 mL of dioxane, and a higher reaction temperature (240 °C) was applied furnishing 30% of the lactam **29#** (entry 15, Table 18).

A few experiments were conducted in order to produce the nootropic agent piracetam from lactone **31a** and glycine amide hydrochloride (**30s**), as depicted in Scheme 12.



Scheme 12. *Attempted synthesis of piracetam.*

Despite the reduction of the reaction temperature and time, the addition of bases and additional cosolvent, these attempts resulted in extensive formation of non-fully characterized side products, and no piracetam was formed in any of the samples. It is possible that the Cl^- ion replaced the BF_4^- as counterion to the ionic liquid, which would lead to significant changes in the properties. For example, [bmim]Cl is much less thermally stable than [bmim]BF_4 (see Table 13).[276]

In most cases, general method **A** was superior to general method **B**, which, however, often resulted in a cleaner reaction mixture implying easier subsequent isolation of the product. In conclusion, these one-pot protocols afford both lactone opening and subsequent lactam cyclization in one oporational step without the addition of a Brønsted acid or base. They have several advantages compared with previously published procedures, such as speed and ease of reaction set-up. The work-up and isolation of products are relatively uncomplicated, due to reduced formation of side products. Several acid-sensitive substrates, e.g. entry 3, Table 16 and entries 4 and 5, Table 17, exemplify the extended substrate range. The ILs [bmim]BF_4 and [bmim]PF_6 were identified as the auxiliary promoting cyclization. Considering the generally high yields, it is unlikely that the IL interferes with the reaction or the substrates.

5 Microwave-Assisted Polymerase Chain Reaction (Paper IV)

The PCR technique has provided a means of producing detectable amounts of DNA from infinitesimal sources of genetic material. Surprisingly, methods of increasing the amount of DNA in one PCR experiment are rare. The factors limiting large-scale PCR have been slow heat transfer and reproducibility. MW-mediated heating has provided organico-chemical applications with low temperature gradients, lack of hot walls and speed, factors highly relevant in sensitive biochemical reactions. A proof-of-principle investigation of MW-assisted PCR was performed by Fermer et al. in 2003.[80] They showed that Taq Pol was stable under MW irradiation and achieved reproducibility in a 50-µL reaction. The thermocycling procedure included MW-heated denaturation and extension, while the annealing was carried out in a water bath.

With the development of more advanced MW reactors allowing parameter programming, the possibility to perform fully automated thermocycling protocols emerged.

5.1 Optimization of Reaction Conditions

The amplified sequence was a 53-bp fragment of human genomic DNA from chromosome 13. All reactions were conducted using 160 nM forward primer 5′-(AGACCTTGGGATACTGCACGG)-3′ and rewind primer 5′-(CCATTCCACAGCCTGGCACT)-3′, 320 µM dNTP, 20 ng/µL BSA and Taq Pol in a buffer consisting of 2.9 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3. The accuracy of the PCR parameters was verified by rt-PCR.

The MW instrument employed allows programming of multistep reactions in which several parameters can be varied in each step, e.g. time, temperature, air-jet cooling, initial input power, maximum power/hold power, maximum pressure/hold pressure. In addition, the absorption level of the reaction mixture can be set to ‘normal’, ‘high’, or ‘very high’. This reduces the risk of over-shooting the temperature threshold, due to slow temperature regulation. The reaction volume range of the instrument is 0.2–20 mL.

5.1.1 Temperature Measurement Control

A PCR experiment should be performed at the lower end of the temperature range for the MW instrument (60–250 °C). As the Taq Pol cannot withstand heating above 100 °C, the accuracy of the temperature measurement of the MW instrument was investigated. The instrument was supplied with a built-in IR pyrometer, which has an error of measurement too great for a biological system. Therefore it was complemented with a fibre-optic (FO) probe, which measured the temperature *in situ* (Figure 22). Nevertheless, the accuracy of the FO probe using boiling pure water was found to be systematically erroneous. Linear regression based on three points, using a temperature cell with a crystallizing compound (26.65 ºC), boiling dry tetrahydrofuran (67 ºC) and boiling pure water (100 ºC), gave the equation $T_{actual} = 0.976 \times T_{reading} + 0.815$, which was used to correct the temperature readings. The standard deviation of the FO probe was determined to be ± 0.3 °C.

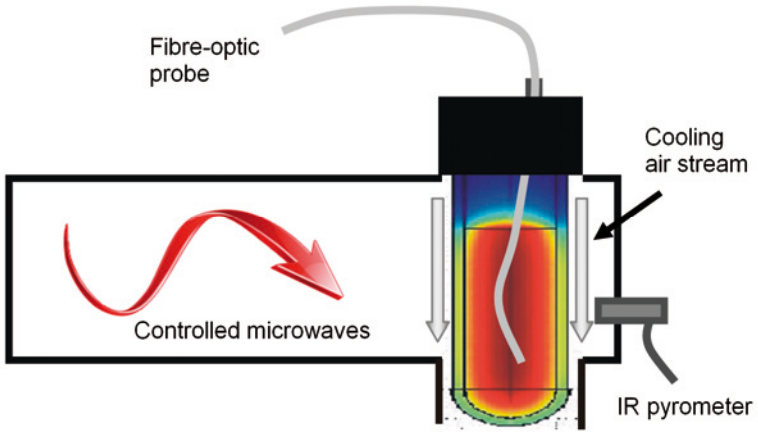


Figure 22. *The set-up in the microwave reactor for the PCR reactions with both fibreoptic and IR pyrometric temperature measurement.*

5.1.2 Programming the Microwave Instrument

In a normal PCR experiment, 30–35 thermocycles are required to reach the plateau phase. The maximum number of operational steps that could be programmed for the MW instrument was 99. Thus, a 33-thermocycle PCR was limited to include three operational steps in each thermocycle. One of these three steps was allocated for air jet-air cooling, hence only two steps of MW-induced heating could be programmed. This required a modification of the classical PCR procedure, in which denaturation, annealing and elongation all take place at different temperatures (Figure 16b). The PCR settings were optimized under conventional heating conditions, with denaturation at 95 °C for 15 s and combined annealing and elongation at 60 °C for 60 s for a 0.05

mL sample. Interestingly, the two-temperature PCR required the addition of more $MgCl_2$ than the normal three-temperature procedure.

The purpose of the initial assays with the MW instrument was to reproduce the heating curve obtained with a conventional PCR thermocycler. The reaction volume was 2.5 mL and PCR was performed without template DNA in the reaction mixture. The aqueous PCR buffer absorbed MWs very efficiently, due to the high ion content. Temperature overshoot, i.e. a temperature higher than the programmed temperature, could be avoided to some extent by setting the absorption level to 'very high'. However, the temperature rise to 95 °C was very slow, leading to a significant risk of deactivation of Taq Pol, and an undesirably long total reaction time. With an initial MW pulse of 300 W, the temperature rose almost instantaneously. The programmed target temperature was decreased in order to reduce the risk of exposing the enzyme to too high temperatures due to overshooting. In the elongation phase, the maximum MW effect was limited to 15 W in order to avoid overshooting.

5.2 Amplification on a mL Scale

Being able to mimic the conventional thermocycle protocol, a 2.5 mL sample including template DNA was processed. The denaturation step of the first thermocycle was adjusted to compensate for the lower starting temperature (i.e. RT). The MW instrument was programmed to heat for 45 s at 90 ºC with 175 W initial power in the very first heating step, cool for 50 s and finally heat for 85 s at 60 ºC with 15 W maximum power during the first thermocycle. The 32 subsequent thermocycles differed in the denaturation step, in that the instrument was programmed to heat for 35 s at 88 ºC. In total, 33 thermocycles were processed in 1 h and 34 min. The resulting temperature profiles obtained using the external IR sensor and the FO probe are shown in Figure 23.

In Figure 23b), three thermocycles have been enlarged to show the expected discrepancy of the IR pyrometer during the cooling step. In fact, the minimum temperature of each cycle was 54 °C and not 46 °C as recorded by the IR pyrometer. The deviation between the two temperature measuring methods was significantly smaller in the other steps of the thermocycles. Despite efforts to reduce overshoot, the maximum temperature recorded during the denaturation step was 92–93 °C.

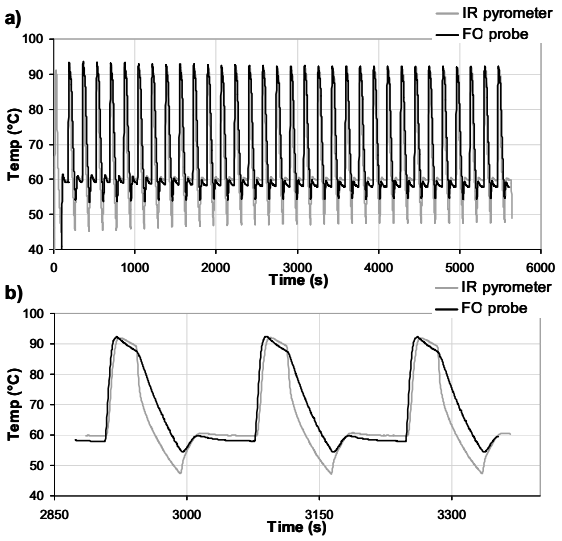


Figure 23. *Temperature profiles of a 2.5-mL PCR experiment. The temperature readings from the external IR pyrometer (grey) and the internal FO probe (black) are shown. a) Temperature profiles for all 33 thermocycles and b) for three representative thermocycles.*

A successful transition to 15 mL was achieved by prolonging the denaturation step to 50 s. The cooling phase of the first two thermocycles was first programmed to be 80 s, but this proved to be insufficient to reach the target temperature of 60 °C and was thus extended to 90 s for the third thermocycle and to 95 s for the remaining 30 thermocycles. In analogy with the 2.5-mL experiment, the settings for the initial heating step were slightly different and included 60 s of heating at 90 °C with an initial MW pulse of 300 W. The readings from the IR pyrometer are shown in Figure 24. The total reaction time for the 15-mL PCR protocol was only 2 h 7 min.

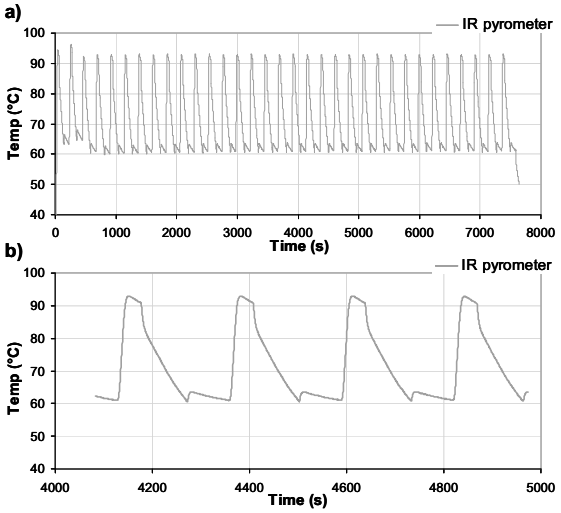
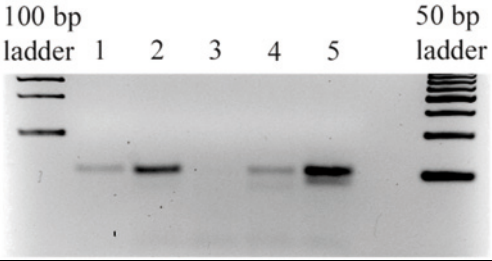


Figure 24. *Temperature profiles from the 15-mL PCR experiment. a) Temperature profiles for all 33 thermocycles and b) for four representative thermocycles.*

The 2.5-mL PCR experiment was repeated, and a negative control sample, prepared and thermocycled under the same conditions, but without the addition of template DNA, was run. Table 19 summarizes the reaction conditions and the outcome of the MW-assisted PCR reactions. The corresponding data for a conventional PCR with a volume of 0.050 mL are given for comparison. The product of all reactions were analysed by GE.

Table 19. *Reaction conditions, amplification results, and gel electropherogram of the PCR experiments.*



Entry	**Description**	**Denaturation**	**Cooling**	**Elongation**	**Amplification efficiency**		
Lane		Temp., Time	Time	Temp., Time	GE	CE	rt-PCR
1	50 µL, conv.	95 °C, 15 s	-	60 °C, 60 s	-	-	-
2	2.5 mL, MW	88 °C, 35 s	50 s	60 °C, 85 s	High	96%	92%
3	2.5 mL, control	88 °C, 35 s	50 s	60 °C, 85 s	None	0%	-
4	2.5 mL, MW	88 °C, 35 s	50 s	60 °C, 85 s	Medium	92%	86%
5	15 mL, MW	88 °C, 50 s	95 s	60 °C, 85 s	High	~100%	98%

conv., Conventional PCR. The colours of the electropherogram have been inverted for clarity.

Lanes 2 and 5, corresponding to one of the 2.5-mL PCRs and the 15-mL PCR, showed very strong bands, whereas the other 2.5-mLexperiment (lane 4) resulted in lower amplification efficiency and a weaker band. No amplification product was detected in the negative control (lane 3). The product from the conventional PCR was purified and served as a size reference in combination with the ladders and the band intensity cannot be compared in respect to amplification efficiency.

The amplification efficiencies of the two 2.5-mL reactions and the 15-mL reaction were revealed by further analysis using CE and rt-PCR, and the results (Table 19) verified the trends seen with GE. The 15-mL PCR proved to be the more efficient, with a calculated yield of each cycle approaching 100% or 98% according to CE or rt-PCR, respectively. There was, however, a side-product, seen as a weaker band below the main product in lane 5. The CE analysis indicated that the side product was about 43 bp long, corresponding to the size of a possible primer-dimer adduct (Figure 25). This concomitant PCR-amplification did not affect the replication of the intended DNA template.

It is most likely that the annealing of the primers to the single-stranded DNA occurred in the late stage of the cooling steps. The IR sensor registered a considerably higher minimum temperature in the 15-mL PCR (60–61 °C) than in the 2.5-mL PCRs (45–48 °C), which could explain the non-specific annealing of the primers.

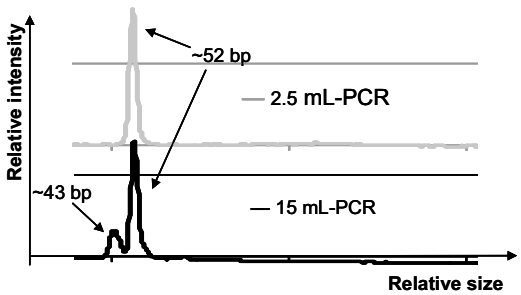


Figure 25. *Capillary electropherograms of a 2.5-mL PCR (grey) and a 1- mL PCR (black). The side product in the 15-mL PCR is the small peak labelled ~43 bp.*

The differences in the temperature profiles could also be the reason for the higher yield in the larger-scale PCR. As the temperature-readings never reached below 60 °C in the 15 mL PCR, probably corresponding to an actual temperature of at least 65 °C, the temperature in the elongation steps probably exceeded 63 °C, and was thus closer to the optimal temperature for Taq Pol. In the 2.5-mL PCR experiments, the corresponding IR reading of temperature never exceeded 61.5 °C.

The only difference between the two 2.5-mL duplicates was the time of day at which they were performed. There are many possible explanations of

the lower yield of the second reaction (entry 3, Table 19), such as contaminated glassware or a higher room temperature, resulting in less efficient air-jet cooling and thus more overshooting in the denaturation setps. This did not seem to affect the Taq Pol in the 15-mL reaction, as the first two thermocycles included temperatures above 95 °C.

In summary, the single-mode MW instrument dedicated for organic synthesis could be used for fully automated PCR thermocycling. The possibility of programming the instrument, not only for temperature and duration of MW irradiation, but also for input power and length of cooling, was crucial for successful amplification. It can also be stated that Taq Pol was not negatively affected by the MW irradiation, regarding stability, selectivity or activity. It has been claimed in several studies that non-thermal effects of MW irradiation can cause irreversible denaturation or impair the functionality of enzymes.[308-311] During the course of this project, it was shown that accurate temperature measurement was crucial for high-yield PCR. The causes of deviating enzymatic activity can not be determined without calibrated temperature-measuring instruments, sufficient stirring and a homogeneous electromagnetic field, guaranteeing the absence of hot spots or cold spots in the sample. No positive effect of the MW irradiation on the enzymatic activity was observed. Nonetheless, it cannot be excluded that Taq Pol is affected by a non-thermal MW effect, such as that observed for Pfu CelB by Young et al.[88] However, in contrast to Pfu CelB, Taq Pol has measurable activity at the entire temperature range of PCR thermocycling (50-96 °C).

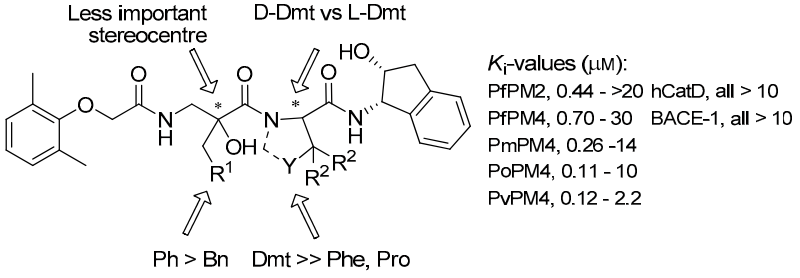
Although no attempts have been made to scale up PCR using microwaves, there is still considerable interest in the methodology,[312] and the concept was recently described in a patent application.[313]

6 Concluding Remarks

This thesis describes the application of MW-induced heating in various areas of drug discovery, more specifically, the enhanced synthesis of novel malaria aspartic protease inhibitors (medicinal chemistry), the expedient formation of saturated lactams, α,β-unsaturated lactams and lactones (heterocyclic chemistry), the fast generation of a small combinatorial library of α,β-unsaturated amides (combinatorial chemistry) and MW-promoted polymerase chain reactions (biochemistry).

The following conclusions can be drawn from the results.

- Twelve diastereomerically pure, peptidomimetic inhibitors of malaria aspartic proteases were synthesized from four epoxy acids and four capped amino acids.
  - The obtained inhibitors incorporate either an α-phenylnorstatine or an α-benzylnorstatine scaffold, both providing a tertiary alcohol as transition-state mimicking group.
  - Some of the inhibitors show activity in the nanomolar range against the plasmepsin active in the digestive vacuole of all four *Plasmodium* species that infect man, i.e. PfPM4, PmPM4, PoPM4 and PvpM4.
  - D-Dmt was discovered as a new interesting P1′ residue.
  - Two inhibitors exhibit inhibition at >1 μM against PfPM2.
  - No inhibitor showed K_i values comparable to those of the lead compound **KNI-10006**.
  - None of the inhibitors exhibited inhibition of the human aspartic proteases hCatD and BACE-1 within measurable limits.
  - New MW-assisted synthetic routes to thiazolidines and epoxy esters were developed.



- Eleven α,β-unsaturated lactams and lactones were prepared in a one-pot, single-operation domino reaction from α- and β-substituted carbonyl compounds with the aid of Bestmann's ylide (Ph_3PCCO) and MW-mediated heating in very short reaction times (5–8 min).

- A small combinatorial library of α,β-unsaturated amides was produced from five aldehydes and five amines in a three-component, one-pot, single-operation domino reaction including Ph_3PCCO and MW heating.

- Twenty-five saturated lactams were synthesized from eight lactones and 17 amines with various substituents in a one-pot, single-operation, two-step domino reaction in ionic liquids under MW irradiation. The absolute configuration of chiral reactants was retained.

- The first fully automated MW-irradiated PCR experiment was developed in a MW instrument dedicated for organic synthesis. The reactions were conducted with large volumes (2.5 mL and 15 mL) and the amplification rates were similar to those in conventional μL-scale PCR.

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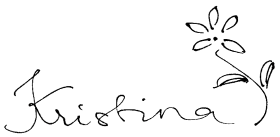

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