Development of new inhibitors for the type II transmembrane serine protease matriptase

Dissertation

zur

Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

dem Fachbereich Pharmazie

der PHILIPPS-UNIVERSITÄT MARBURG

vorgelegt von

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Marburg/Lahn 2012

Vom Fachbereich Pharmazie der Philipps-Universität Marburg

als Dissertation angenommen am: 22.08.2012

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Tag der mündlichen Prüfung: 23.08.2012

Die Untersuchungen zur vorliegenden Arbeit wurden auf Anregung von Herrn Prof. Dr. Steinmetzer am Institut für Pharmazeutische Chemie des Fachbereichs Pharmazie der Philipps-Universität Marburg in der Zeit von Januar 2008 bis May 2012 durchgeführt.

Dedication to my family

For the sacrifices they made, their love, and support

Contents

Abbreviations

The abbreviations of the amino acids and their derivatives are written according to the recommendations of the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN) using the three or one letter code. Unless otherwise indicated, all amino acids and their derivatives have L-configuration. Abbreviations of chemical compounds are taken from the relevant references like Methods of Organic Chemistry (Houben-Weyl), March's Advanced Organic Chemistry and Greene's Protective Groups in Organic Synthesis.

Å	Ångstrom (1Å = 10^{-10} m)
Ac	acetyl
HOAc	acetic acid
ACN	acetonitrile
Am	amidine
AcOxAm	acetylhydroxyamidine
Amba	4-amidinobenzylamide
Abu	α-aminobutyric acid (homoalanine)
Bzls	benzylsulfonyl
Boc	*tert.*-butyloxycarbonyl
Bzlu	benzylurea
calcd	calculated
Cbz	benzyloxycarbonyl
Chxu	cyclohexylurea
Dbzg	dibenzylglycine
DIPEA	diisopropylethylamine
Dpheg	diphenylethylglycine
$(Boc)_2O$	di-*tert*-butyl dicarbonate
DCM	dichloromethane
DMF	*N*,*N*-dimethylformamide

DMSO	dimethylsulfoxide
Et	ethyl
EtOAc	ethyl acetate
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
equiv	equivalent
EtO	ethoxy
fXa	factor Xa
Fig.	figure
h	hour
OxAm	hydroxyamidine
HAT	human airway trypsin-like protease
HATU	O-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
hPhe	homophenylalanine
hTyr	homotyrosine
K_i	dissociation constant for the enzyme inhibitor complex
K_m	Michaelis-Menten-constant
MMPs	matrix metalloproteinases
MeOH	methanol
MeO	methoxy
MS	mass spectrometry
n.d.	not determined
NMM	*N*-methyl morpholine
NMR	nuclear magnetic resonance spectroscopy
Nva	norvaline
Pbf	2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl
Pd/C	palladium on activated charcol
Phg	phenylglycine

Phe(3-CN)	3-cyanophenylalanine
PDB	protein data base
Pip	piperidide
pNA	*para*-nitroanilin
ppm	*parts per million*
PAR-2	proteinase-activated receptor 2
Pra	propargylglycine
PyBOP	benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
Pyr	pyridyl
R_f	retardation factor (TLC)
RP-HPLC	reversed phase-high performance liquid chromatography
RT	room temperature
tBu	*tert.*-butylester
TEA	triethylamine
TFA	trifluoroacetic acid
Tfa	trifluoroacetyl
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMPRSS2	transmembrane protease serine 2 (epitheliasin)
TMS	trimethylsilane
Tris	tris(hydroxymethyl)aminomethane
TTSP-II	type II transmembrane serine protease
uPA	urokinase-type plasminogen activator
V_{max}	maximum velocity of non-inhibited enzyme reaction
Cbz-OSu	*N*-(benzyloxycarbonyloxy)succinimide

1 Introduction

1.1 Cancer

Cancer is medically known as a malignant neoplasm, which is a group of diseases characterized by uncontrolled growth and the spread of abnormal cells. It may also extend to more distant parts of the body through the lymphatic system or bloodstream. Cancer is caused by a multistep process of alterations in several oncogenes due to either the damage of genes or to genetic faults within cells in tumor-suppressor genes and microRNA genes. Oncogenes encode proteins that control cell proliferation, apoptosis, or both. They can be activated by structural alterations resulting from mutation or gene fusion.[1, 2] Oncogenes produce six broad groups of products: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers, and apoptosis regulators. These products are involved in the initiation and progression of tumors and have become important targets for the development of new anticancer drugs.[2] The alterations in genes occurred as a result of internal factors, such as inherited mutations, hormones and immune conditions; and environmental/acquired factors such as tobacco, diet, radiation, and infectious organisms, (Fig. 1.1). The contribution of genetic factors and environmental factors towards cancer risk is 5–10% and 90–95%, respectively. The evidence indicates that of all cancer-related deaths, almost 25-30% are caused by tobacco, as many as 30-35% are linked to diet, about 15-20% are due to infections, and the remaining percentage are due to other factors like radiation, stress, physical activity, and environmental pollutants, etc.[3]

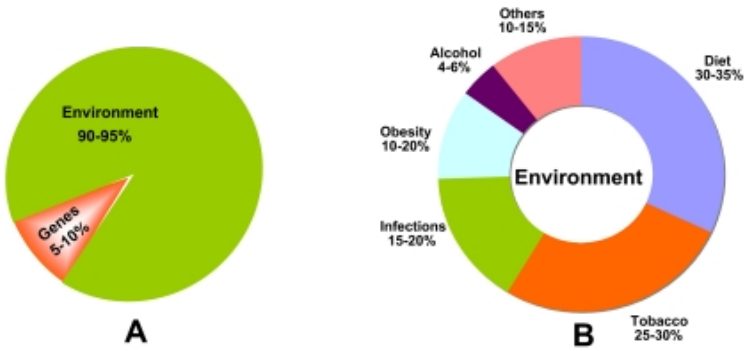


Fig. 1.1 The role of genes and environment in the development of cancer. A: The percentage contribution of genetic and environmental factors to cancer. B: Percentage contribution of each environmental factor. The percentages represented here indicate the attributable-fraction of cancer deaths due to the specified environmental risk factor (figure adapted from Anand, 2008[3]).

The international agency for research on cancer (IARC) considered cancer as one of the leading causes of death in economically developed countries and the second leading cause of death in developing countries.[4] About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide, with 56% of the cases and 64% of the deaths in the countries of the third world. Breast, lung, colorectal, and cervix cancers in females and lung, liver, colorectal, and prostate cancers in males are the most frequently diagnosed cancers and the leading cause of cancer death worldwide.[5] Cancer cells possess a broad spectrum of migration and invasion mechanisms. These include both individual and collective cell-migration strategies. The ability of a cancer cell to undergo migration and invasion allows it to change position within the tissues. For example, cell-migration processes allow neoplastic cells to enter lymphatic and blood vessels for dissemination into the blood stream, afterwards the cells undergo metastatic growth in distant organs.[6] To spread within the tissues, tumor cells use migration mechanisms, where the cell must modify its shape and stiffness to interact with the surrounding tissue. Hereby, the extracellular matrix (ECM) provides the substrate, as well as a barrier towards the advancing cell body. Proteases in general, including type II transmembrane serine proteases (TTSPs), play a potential role in degrading components of the ECM and thus give tumor cells the ability to migrate and spread within living tissues. In recent years, numerous studies focused on the expression of individual TTSPs including matriptase during tumor progression; and on the possible functions of these enzymes in tumor cell proliferation, migration and invasion.[7] For these reasons, inhibition of such enzymes in the initiating steps of cancer might have clinical importance in tumor development and this offers a new rational strategy for cancer treatment.

1.2 Proteases and their roles in tumorgenesis and tumor suppression

Proteases are a class of enzymes that play fundamental roles in multiple biological processes and are associated with a wide variety of pathological conditions, including cancer. They act as processing enzymes, catalyze the cleavage of specific substrates and influence cell behavior, survival and death.[8] In the 1970s various proteases of the serine, cysteine, and metalloproteases families with pro-metastatic activities began to be identified. Intracellular and extracellular proteases can operate as signaling molecules in various cellular processes that are essential for cancer biology. These protease-regulated

processes include proliferation, adhesion, migration, differentiation, angiogenesis, senescence, autophagy, apoptosis and immune response. In addition, proteolytic processing is necessary in nearly every stage of cancer growth and progression, from angiogenesis to extracellular matrix remodeling, cell-to-cell signaling, and metastasis.[9, 10]

Intracellular proteases

In many cases the function of intracellular proteases in signaling cascades is associated with the removal of damaged or undesirable products, e.g., lysosomal cysteine and aspartyl cathepsins mediate the degradation of endocytosed proteins.[11] The intracellular cysteine proteases of the caspase family play a role in regulation of proteolytic activities that result in apoptosis.[12, 13] For instance, CASP8 is found to be inactivated by somatic mutations in malignancies including head, neck, lung, and gastrointestinal carcinomas. This might represent an additional strategy for cancer progression. In addition, the loss of CASP8 was found to increase the risk of metastasis in neuroblastoma patients. That result has led to the assumption that this protease is a metastasis suppressor that regulates the survival and invasive capacity of neuroblastoma cells.[14, 15] Another cysteine protease family, the autophagins, contribute to the 'self-eating' effect that is found during starvation conditions,[16] and deubiquitylases (DUBs) are responsible for removing ubiquitin from ubiquitinylated proteins and peptides.[17, 18]

Extracellular proteases

Extracellular proteases belong to different clans including metalloproteases such as MMPs, ADAMTSs (a disintegrin and metalloproteinases with thrombospondin domains) and neprilysin (MME), the cysteine cathepsins, and several serine proteases like kallikreins, testisin (PRSS21), prostasin (PRSS8) and dipeptidyl peptidase-4 (DPP-4).[19] These extracellular proteases are thought to be actively involved in facilitating tumorigenesis since they promote tumor progression and metastasis. They are frequently overexpressed in malignant tissues. This aspect led to the development of small-molecule inhibitors for the treatment of cancer, in particular of molecules targeting matrix metalloproteinases (MMPs) and plasminogen activators.[8] By contrast, other clinical studies have shown in some cases that the inhibition of metalloproteinases causes an acceleration of tumor growth. This suggests that some extracellular and pericellular proteases of all major catalytic classes might have anti-tumor roles and

suppress tumor progression. MMPs have long been associated with cancer invasion and metastasis owing to their ability to degrade the extracellular matrix.[10] However, recent studies have shown that several members of this family, including collagenase 2 (MMP8), macrophage metalloelastase (MMP12) and matrilysin 2 (MMP26) provide a protective effect in different stages of cancer progression. Furthermore, other MMPs such as stromelysin 1 (MMP3), gelatinase B (MMP9), stromelysin 3 (MMP11) and MMP19, which were originally recognized as pro-tumorigenic proteases, might also function as protective enzymes in some specific situations.[10, 20] Examples of the extracellular serine proteases with tumor suppressing activity are testisin and prostasin which are members of the glycosylphosphatidylinositol (GPI)-anchored proteases with trypsin-like activity. Prostasin can suppress tumor growth and invasion of prostate and breast cancer cells,[21] e.g., it is downregulated in prostate cancer.[22] Furthermore, the ectopic expression of testisin in testicular tumor cells was found to suppress their tumorigenicity,[23] although opposing results appeared in ovarian cancer cells.[24] Urokinase, a serine protease that is frequently associated with cancer progression, might also delay tumor development in specific cancer models.[25] Dipeptidyl peptidase-4 (DPP-4) is a cell surface serine protease that was first reported to suppress the malignant phenotype of melanocytic cells,[26] and it has subsequently been found to have protective functions for different human malignancies.[27] By contrast, DPP-4 inhibitors a class of oral hypoglycemic drugs, such as sitagliptin or vildagliptin have been in clinical use for the treatment of diabetes mellitus type 2 for several years.

Moreover, several other serine proteases that are generally associated with cancer progression have been reported to have a protective function in specific instances. These examples highlight the dual roles of some proteases, depending on the cellular source and cancer microenvironment.[7, 28]

1.3 Trypsin-like serine proteases

The family of trypsin-like serine proteases consists of approximately 70 members. These enzymes have been found to play a major role in a variety of physiological processes including digestion, blood coagulation, regulation of blood pressure, fibrinolysis, pathogen-host interaction, wound healing, immune responses, tumor growth, invasion, and metastasis, and they have been implicated in the etiology of a

number of human diseases related to these systems.[29] In recent years, subgroups of these enzymes have been identified, which are anchored directly to plasma membranes, either by a carboxy-terminal transmembrane domain (Type I), an amino-terminal transmembrane domain with a cytoplasmic extension (Type II or TTSP), or through a GPI linkage. An additional mechanism is used by uPA (urokinase), which is fixed to the cell surface via binding to a specific uPA-receptor.[30] These trypsin-like serine proteases have a common catalytic mechanism for the cleavage of specific substrates and are frequently involved in consecutive proteolytic reactions or protease cascades, where one protease precursor (zymogen) is the substrate of an active protease. This shared mechanism confers the advantage that a single signal may be specifically and irreversibly amplified at each step, when a downstream zymogen is activated, providing the capacity for unleashing a burst of proteolytic potential.[31]

1.4 Type II transmembrane serine proteases

TTSPs are serine proteases defined by a hydrophobic single-pass transmembrane domain located near the amino terminus of the protein that separates a short intracellular domain from a larger extracellular portion. The extracellular part of the molecule contains a highly variable stem region and a carboxy-terminal trypsine-like serine protease domain of the chymotrypsin S1 fold.[32-34] Localization to the cell surface gives these enzymes an excellent opportunity to mediate signal transduction between the cells and their extracellular environment, and to regulate various cellular responses.

The TTSPs have been divided into four subfamilies based on the phylogenetic analysis of the serine protease domains and the domain structure of the extracellular stem region. These are the human airway trypsin-like protease HAT/DESC-, the Hepsin/TMPRSS-, the Matriptase- and the Corin-subfamilies. Their schematic structures are shown in Figure 1.2.[32, 33, 35] The first described TTSP; enteropeptidase has an essential role in food digestion and was identified over a century ago by Pavlov. In 1994 the cloning of the enteropeptidase cDNA has revealed the presence of a membrane-anchor and explained its localization to the plasma membrane.[36] Meanwhile, 19 active members of TTSPs have been identified in mice and humans, which have a similar modular structure as found in enteropeptidase. They were also found in all vertebrate species and in the non-vertebrate species Drosophila.[37] Several members of the family have already been assigned important roles in the normal development of human or mouse

tissues. Additionally, many of the TTSPs are consistently overexpressed in a great variety of tumors, suggesting their potential as novel tumor markers and targets for anti-cancer therapy.[35]

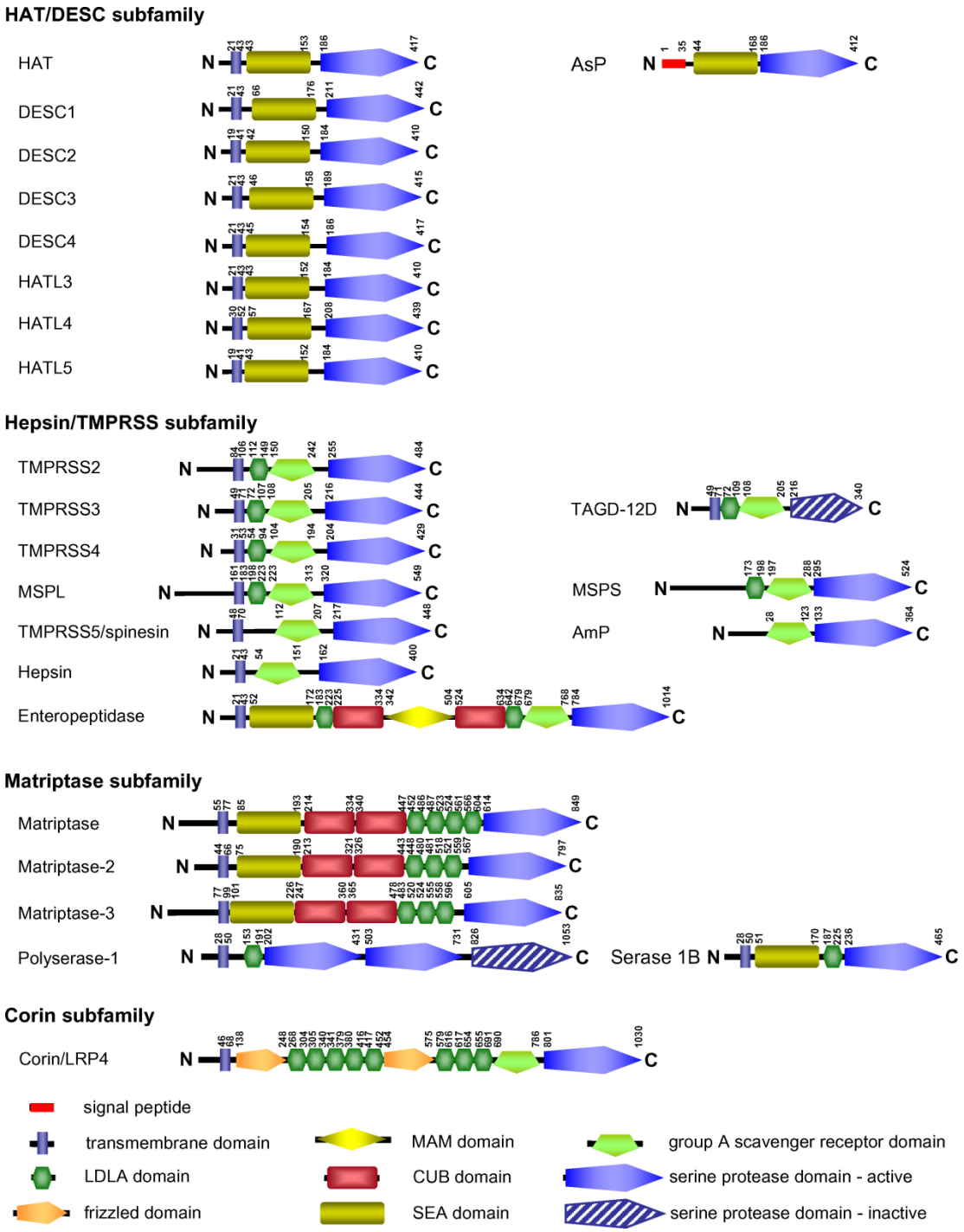


Fig. 1.2 Predicted domain structures of human type II transmembrane serine proteases. Numbers indicate the position of each domain in the amino acid sequence of native, pro-enzyme molecules (figure adapted from Szabo and Bugge, 2008[28]).

All members of the four TTSPs subfamilies are synthesized as single chain zymogens that are activated by proteolytic cleavage after a specific arginine or lysine residue within a highly conserved activation motif preceding the catalytic domain. Several of the biochemically purified TTSPs, such as HAT, matriptase, matriptase-2, hepsin and TMPRSS2 to 4 rapidly undergo autocatalytic activation *in vitro*.[32]

All TTSPs contain six conserved cysteine residues within their catalytic domains that are predicted to form three intradomain disulfide bonds, as known for all serine proteases domain of the S1 fold. TTSPs exhibit a strong preference for substrates that contain an Arg or Lys residue in the P1 position and they may be activated by other members of the family or be subject to intermolecular autoactivation *in vivo*.[35] After activation, the C-terminal protease domain remains covalently linked to N-terminal located domains by a conserved disulfide bond and can develop their activity in cellular compartments or on the cell surface.[32]

Of all TTSPs, the HAT/DESC subfamily exhibits the simplest modular structure of the stem region (Fig. 1.2), which consists only of a SEA domain (single sea urchin sperm protein, enteropeptidase, agrin domain).[8, 38] The function of this segment is currently unknown, but there are indications that the SEA domains are associated with glycosylation reactions, and it is known that an additional autocatalytic (proteolytic) cleavage in this region of matriptase (see section 2.2) and enteropeptidase is essential for subsequent activation of the protease.[39, 40] The SEA domain-containing TTSPs were found to be released by a second cleavage within the SEA region from the cell surface.[41, 42] This cleavage within the SEA domain allows the shedding of the protease from the cell surface where soluble forms of enteropeptidase, HAT, TMPRSS2, and matriptase have been detected *in vivo*.[43, 44] These shed forms may be involved in the regulation of TTSP activity and function. The hepsin/TMPRSS/enteropeptidase subfamily includes hepsin, MSPL, TMPRSS2–5 and enteropeptidase. With the exception of enteropeptidase, members of this subfamily exhibit a relatively short stem region containing a SRCR domain (a single scavenger receptor Cys-rich, also abbreviated as SR domain), either alone as in hepsin and TMPRSS5 or proceeded by an LDLA domain (a single low density lipoprotein receptor class A) in TMPRSS2–4 and MSPL. Furthermore, enteropeptidase displays a unique structure with a complex stem region consisting of a SEA domain following the signal anchor, a SRCR domain preceding the serine protease domain, and two LDLA domains separated by two CUB

domains (bone morphogenic protein 1), and a MAM domain (a meprin, A5 antigen, and receptor protein phosphatase μ). In corin, the only member of the corin subfamily, two frizzled, eight LDLA, and an SRCR domain were identified in the stem region (Fig. 1.2).[29, 33] The specific contributions of each stem domain to TTSP proteolytic activities have not yet been well defined, although for many TTSPs, the stem domain is required for efficient cleavage of their physiological substrates.[8]

Physiological and pathophysiological functions of the TTSPs

Many TTSPs show relatively strict patterns of expression and were detected in different tissues and cells of the human body. For example, enteropeptidase is expressed on the apical surface of enterocytes and goblet cells in the proximal small intestine and is the principle trypsinogen activator.[44] Hepsin is mainly found in hepatocytes, while its expression has been identified in a number of additional tissues including thymus, thyroid, lung, pancreas, pituitary gland, prostate, and kidney, as well as in cultured human endothelial cells grown in 3D type I collagen matrices.[45, 46] It is thought to play a role in the growth of some cells as well as potently activating the proform of hepatocyte growth factor (HGF)[46] and pro-uPA.[47] Corin is highly expressed in cardiomyocytes and converts pro-ANP and pro-BNP (atrial and brain natriuretic peptides, cardiac hormones that regulate blood pressure and cardiac function by promoting natriuresis, dieresis, and vasodilation) into smaller biologically active molecules.[48, 49] HAT is mainly localized in human trachea and bronchi, and its upregulation is found to be associated with inflammatory environments. It also cleaves the surface glycoprotein hemaglutinin (HA) of the human and avian influenza viruses of subtypes H1, H2, and H3 at a monobasic cleavage site, which is a prerequisite for influenza virus replication.[50, 51] HAT has been demonstrated to have the capacity to degrade fibrinogen to activate pro-uPA to activate membrane receptors such as PAR-2.[52, 53] However matriptase is an exception as it is not restricted to any particular organ. An overview of these proteases expression patterns and their physiological functions were described and summarized in several review articles.[7, 28, 33]

Pathophysiologically, some TTSPs are highly expressed during tumor progression and play a major role in tumor cell proliferation, migration and invasion. For example, an increased expression of hepsin, TMPRSS2 and matriptase was detected in various tumor tissues. Hepsin is involved in the pathogenesis of metastatic prostate cancer,[54]

ovarian and renal cancers. The overexpression of hepsin was an unfavorable clinical forecast in those tumors. Other members of the hepsin/TMPRSS subfamily, TMPRSS2, TMPRSS3 and TMPRSS4 are overexpressed in the epithelium of most prostate cancers, ovarian cancers and pancreatic tumors respectively.[55, 56] Matriptase is involved in the development of a variety of tumors as discussed in section 1.5.[57] Other members of TTSPs have not been clearly associated with cancer, while it should be noted that some members like matripatse-2 seem to have a tumor suppressing function.[58]

1.5 Matriptase subfamily

The matriptase subfamily of the TTSPs consists of three homologous proteases, matriptase, matriptase-2, matriptase-3, and a protein with atypical mosaic structure, polyserase-1. All matriptases have a similar stem region with one SEA, two CUBs, and three (matriptase-2 and matriptase-3) or four (matriptase) LDLA domains (Fig. 1.2). In polyserase-1, the transmembrane domain is followed by a single LDLA domain and a parallel repeat of three serine protease domains, referred to as serase-1, -2, and a catalytically inactive serase-3.[7, 59] Recently, polyserase-2 and -3, two closely related proteases with a similar structure, have been found in mice and humans, but both proteins lack the transmembrane domain and appear to be secreted.[60]

The first identified member of this subfamily is matriptase (MT-SP1), which is found in the epithelial compartments of most embryonic and adult tissues. Several studies have reported the properties and physiological functions of matriptase, which is described in section 1.5.1.

Matriptase-2 (MT2), also known as TMPRSS6, is predominantly expressed in adult and fetal liver tissues in humans and mice with minor expression in the kidney, uterus, and nasal cavity. It has proteolytic activity toward various macromolecular substrates.[61, 62] MT2 suppresses the expression of the hepatic hormone hepcidin, the main regulator of systemic iron homeostasis, through cleavage of the bone morphogenetic protein co-receptor hemojuvelin, which is a key regulator of hepcidin gene activation.[63-65] Otherwise, humans with MT2 deficiency suffer from low iron levels and severe microcytic anemia.[66, 67] Due to the important role of MT2 in iron homeostasis, the enzyme represents a novel target for the development of inhibitors, potentially useful in the treatment of the systemic iron overload (hemochromatosis). In contrast to matriptase, the expression of MT2 correlates with the suppression of the invasiveness

and migration of breast and prostate cancer cells.[58] However, the precise functions of MT2 in cancer remain to be further elucidated.

Matriptase-3, also named TMPRSS7, revealed expression in brain, skin, eye, salivary gland, and the reproductive tissues, including prostate, testis, epididymis, ovary and uterus. The recombinant catalytic domain of matriptase-3 can hydrolyse synthetic peptide substrates with a strong preference for arginine in the P1 position, and shows proteolytic activity toward several macromolecular substrates, including gelatin, casein, and albumin.[68] Studies in animals or humans with matriptase-3 deficiency have not been reported and its physiological function remains to be determined.

The polyserase-1, also named TMPRSS9, was detected predominantly in skeletal muscle, heart, kidney, liver, placenta, and brain.[60] In addition, a shorter splice variant containing only the first of the three serine protease domains of polyserase-1, termed serase-1B (Fig. 1.2), has been described in mice and humans, with its highest expression detected in liver, small intestine, pancreas, testis, and peripheral blood.[40] Matriptase-like enzymes have proteolytic activity against a number of polypeptide substrates *in vitro*, including casein, albumin, pro-uPA, and components of the extracellular matrix such as fibronectin, fibrinogen, and denatured type I collagen, but their functional characterization still awaits further studies.[7, 40, 68]

1.5.1 Identification of matriptase and its catalytic domain

Matriptase is also known as membrane-type-serine-protease 1 (MT-SP1),[69] tumor-associated differentially expressed gene-15 (TADG-15),[70] suppressor of tumorigenicity 14 (ST14), and as epithin for the mouse analogue.[71] It is an 80- to 90-kDa cell surface glycoprotein with a complex modular structure. MT-SP1 was first described and isolated by the Dickson group in 1993 with a new gelatinolytic activity in cultured breast cancer cells.[72] It is widely expressed in various epithelial compartments of many embryonic and adult tissues and in immune cells including mast cells, monocytes and macrophages.[73, 74] Matriptase has a small cytoplasmic N-terminus, followed by a short transmembrane segment and a large extracellular part containing the protease domain on the C-terminus. It is synthesized as an inactive, single chain zymogen and its activation is a multistep process.[75] This activation requires two sequential endo-proteolytic cleavages; the first is after Gly149 within the amino-terminal SEA domain, which occurs in the endoplasmatic reticulum or Golgi apparatus. The SEA domain

cleaved matriptase is associated with HAI-1 (hepatocyte growth factor activator inhibitor-1) and is transported in its complexed form to the cell surface. The second cleavage is an autocatalytic process, which occurs in the serine protease domain between Arg614 and Val615 within a highly conserved activation cleavage site R↓VVGG. This autoactivation appears to be controlled by the stem region, by posttranslational modifications and the cellular localization of the protease.[75] However, the specific mechanisms that trigger the activation of matriptase are incompletely understood.

The structure of the catalytic serine protease domain of matriptase is similar to other proteases of the chymotrypsin family (S1) and is closely related to numerous well know trypsin-like serine proteases, such as thrombin and factor Xa. Therefore, the common chymotrypsinogen numbering will be used for describing the structure of matriptase in the following part. Meanwhile, various X-ray structures of matriptase in complex with benzamdine, the Kunitz-type bovine pancreatic trypsin inhibitor (BPTI), two synthetic 3-amidinophenylalanine-derived inhibitors, and the sunflower trypsin inhibitor-1 have been solved. This information can facilitate the design of new matriptase inhibitors.[76-78] Matriptase contains a Ser190 adjacent to the Asp189, which makes the S1 pocket slightly more hydrophilic and narrow than in other trypsin-like serine proteases containing an Ala190, such as thrombin or factor Xa.[79] The S1 pocket binds basic residues, such as Lys or Arg as found in several substrates or Arg-mimetics used in synthetic inhibitors. The matriptase residues 214-220 and 189-195 on the wall of the S1-pocket are very similar to the sequences of other trypsin-like serine proteases and differ mainly in position 217 and 192. However, the side chain of Asp217 is directed away from the S1 pocket. The S2 site is well suited to accommodate small to medium-sized P2-amino acids such as serine, which was found to be a preferred P2 residue in studies using a combinatorial substrate library prepared by phage display technique.[80] However, the bulky Phe99, which was expected to restrict the access of sterically demanding P2 amino acids, possesses some flexibility and its side chain is significantly rotated within the BPTI complex compared to the X-ray structure found with benzamidine.[77] Therefore, matriptase also cleaves substrates with larger P2 amino acids with high efficiency, such as Phe in pro-uPA or Leu in pro-HGF.[75]

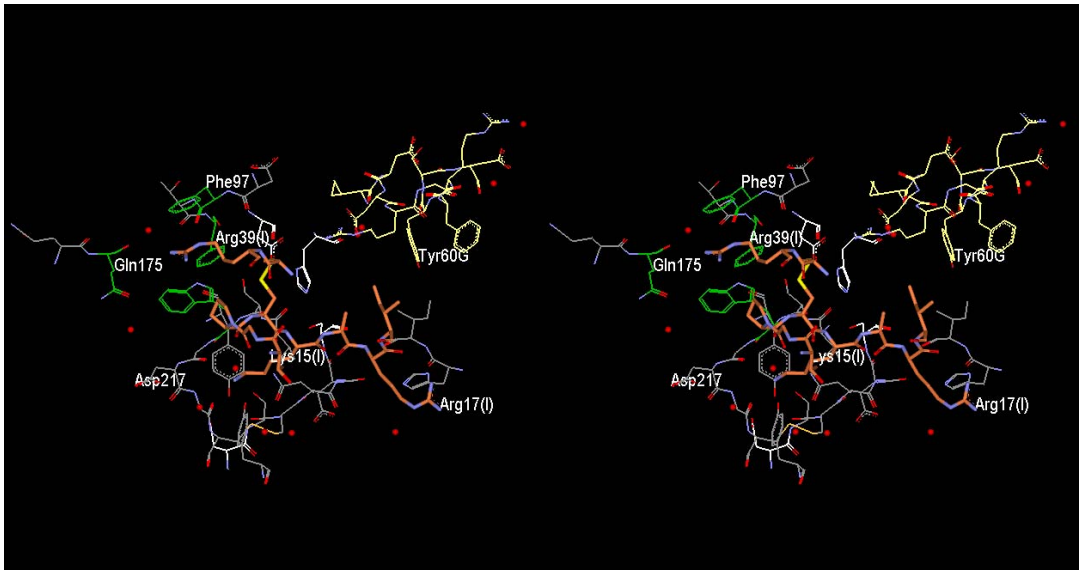


Fig. 1.3 Stereo view of matriptase in complex with BPTI (1eaw.pdb).[77] Shown BPTI residues Thr11(I)-Gly12(I)-Pro13(I)-Cys14(I)-Lys15(I)-Ala16(I)-Arg17(I)-Ile18(I) are disulfide bridged to residues Cys38(I)-Arg39(I) and given as sticks with orange carbon atoms. Matriptase residues His57, Asp102, Ser195 (catalytic triade) and Asp189 at the bottom of the S1-site are colored with white carbon atoms. The amino acids surrounding the S3/4 site (Phe97, Phe99, Gln175 and Trp215) and the amino acids of the 60-insertion loop are shown in green and yellow, respectively (figure modified and adapted from Friedrich, 2002[77]).

Matriptase possesses a characteristic S3/4-binding site, formed by 3 aromatic amino acids, Phe97 on the top, Trp215 at the bottom, surrounded by Phe99 and Gln175. This region is reminiscent of the corresponding pocket found in factor Xa that can accommodate hydrophobic residues, but it can also act as a cation binding site. Probably, both cation/π-interactions and an overall negative potential formed by the carbonyl groups of amino acids 96-98 and the side chain carbonyl of Gln175 contribute to the preferred binding of basic amino acids to this binding site, such as Arg39(I) in the matriptase/BPTI complex (Fig. 1.3).

Matriptase features a unique nine-residue 60-insertion loop with characteristic amino acids surrounding it, as shown with yellow carbon atoms in figure 2.1. A 60-insertion loop exists also in thrombin; however the segment Tyr60A-Pro60D-Pro60C-Trp60D forms a well defined S2 pocket, which contributes to the preference of proline as P2 residue in thrombin inhibitors and substrates.[81] By contrast, the 60-insertion loop in matriptase is rotated away and forms a more open active site. These differences in the structures with the large BPTI and small benzamidine revealed that this 60-insertion loop has some conformational flexibility and can adapt to the bounded ligand.

1.5.2 Potential matriptase substrates in cancer biology

The substrate specificity of MT-SP1 was determined using a positional scanning-synthetic combinatorial library and substrate phage techniques. The preferred P4-P1' cleavage consensus sequences were found to be (Arg/Lys)-Xaa-Ser-Arg↓-Ala and Xaa-(Arg/Lys)-Ser-Arg↓-Ala, where Xaa is a non-basic amino acid.[80] Matriptase is also able to cleave a series of synthetic chromogenic or fluorescent tripeptide substrates with Arg-*para*-nitroanilide or Arg-7-amido-4-methylcoumarin as P1-P1' segment, which can be used for convenient activity measurements.[82] Through many approaches it was demonstrated that matriptase cleaves and activates the proforms of hepatocyte growth factor (HGF), the G-protein-coupled protease-activated receptor 2 (PAR-2) and the soluble urokinase-type plasminogen activator (uPA).[80, 83, 84] Matriptase can also efficiently activate receptor-bound pro-uPA on the surface of cultured monocytes and human ovarian cancer cells, and thereby act as an initiator of the plasminogen activation system. Activated bound uPA promotes the extracellular proteolysis by generating the broad spectrum protease plasmin. Plasmin is also a principal activator of MMPs and involved in the degradation of extracellular matrix proteins (ECM). By contrast to the initiation of the uPA/plasmin/MMP cascade, a direct matriptase-catalyzed activation of stromelysin (MMP-3) was shown *in vitro*.[85] In addition, the uPA/uPAR complex elicits a number of cellular responses that include cellular adhesion, differentiation, proliferation, and migration in a non-proteolytic fashion.[86, 87] The components of the plasminogen activation system are upregulated in several tumor entities and promote tumor invasion and metastasis; therefore, the development of specific uPA inhibitors is activative strategy for the prevention of metastasis.[88-90]

Matriptase converts pro-hepatocyte growth factor/scattering factor (HGF/SF) to its active form,[84] which binds with high affinity to the receptor tyrosine kinase c-Met. Activated c-Met induces multiple signaling pathways leading to the activation of gene products required for tumorigenesis and invasive growth (Fig. 1.4).[57, 91, 92] It has been demonstrated that the inhibition of the HGF binding to its receptor by antagonists or antibodies strongly reduces tumor growth and metastasis.[93-95] Pro-HGF activation by matriptase has been studied in prostate and colon carcinoma cell lines, where inhibition of endogenous matriptase by small interfering RNAs or synthetic inhibitors impaired the conversion of pro-HGF to HGF on the cell surface. It was demonstrated that matriptase processes the inactive form of HGF as efficiently as HGFA and that these two proteases

are more active than other pro-HGF convertases such as uPA, tPA, a serum protease homologous to the coagulation factor XIIa, factor XIIa itself, coagulation factor XIa, or plasma kallikrein, which also activate HGF/SF.[96]

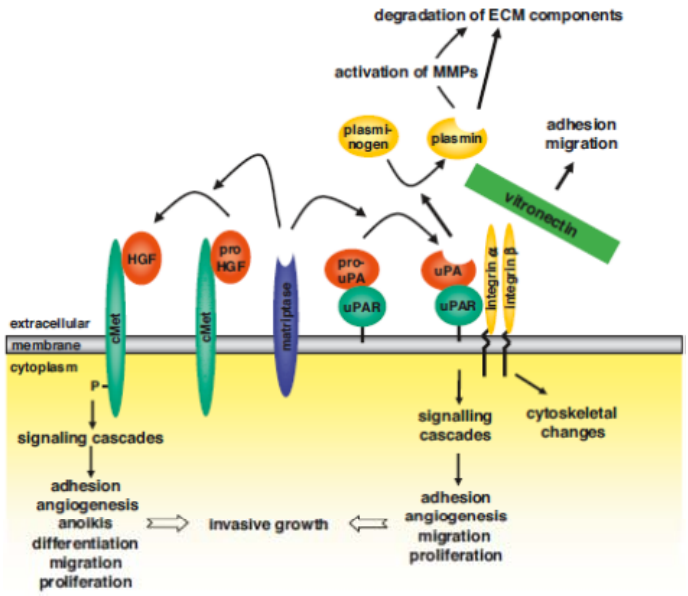


Fig. 1.4 Proposed functions of matriptase in invasive growth. Schematic drawing of the two major pathways that are hypothesized to mediate the pro-invasive effects of matriptase (figure adapted from K. Uhland, 2006[57]).

An additional candidate substrate of matriptase is the G-protein coupled protease-activated receptor 2 (PAR2), which is localized on the extracellular surface.[80, 83] PAR2 mediates cell adhesion, cell mobility and inflammation and has been suggested to stimulate metastasis.[97] However, in addition to matriptase, other proteases such as trypsin, tryptase or factor Xa have been described as potential PAR2 activators.[98]

Matriptase is found to be an efficient activator of prostasin, a glycosylphosphatidyl-inositol-linked membrane serine protease, also abbreviated as CAP1/PRSS8. The two proteases colocalize in the epidermis and in a variety of other epithelia. Since pro-prostasin is incapable of autoactivation, it is likely that matriptase may be a candidate activator of its zymogen in various physiological settings.[99] *In vitro*, matriptase has the ability to convert the prostasin zymogen into the active two-chain form, even though its cleavage sequence (Ile-Gln-Pro-Arg↓Ile in mice) lacks the second basic amino acid in P3 or P4, which is often supposed to be a prerequisite for matriptase substrates.[100]

Another study has shown that the secreted form of matriptase produced in COS-1 cells digested fibronectin and laminin, both are proteins of the ECM and involved in cell

adhesion via binding to specific integrin receptors. This finding suggests that matriptase participates in the control of epithelial turnover by regulating the cell-substratum adhesion.[101]

Recently, it was shown that several human cell lines and also a purified soluble form of matriptase can process the single-chain of insulin-like growth factor (IGF) binding protein related protein-1 (IGFBP-rP1) to a two-chain form.[102] The IGFBPs regulate cellular proliferation by modulating the effects of insulin and IGF,[103] whereas the IGFBP-rPs have low affinity to IGFs/insulin and low structural homology to IGFBPs.[104] Several groups demonstrated tumor-suppressive activity for IGFBP-rP1 *in vivo*, although its exact biological function remains to be clarified.[105-107]

The above-mentioned substrates represent proteins with diverse functions. Future studies accessing their physiological relevance as matriptase substrates will provide information about the importance of matriptase in cancer biology.

1.5.3 Function of matripatse in the development of epidermal tissues

Recent studies suggest that the matriptase and prostasin cascade plays an important role in the development of several tissues. It contributes to the epidermal tissue differentiation, the formation of follicular structures and to the function of skin.[75, 108, 109] The close relation between both proteases is proved in part by the identical phenotypes of matriptase- and prostasin-deficient mice skin, and by the absence of active prostasin in a matriptase-deficient epidermis.[100, 110]

Follicular structures and skin functions were found to be affected by matriptase loss, as shown by generalized hypoplasia of pelage hair follicles of null mice. These phenotypes were linked to defects in the initiation matriptase mediated, caspase 14, calpain I and bleomycin processing the hydrolase of the epidermal polyprotein profilaggrin.[111] Profilaggrin is processed into filaggrin monomer units and subsequently, into free hygroscopic amino acids, which partake in the formation of the cornified envelope of the uppermost layers of the epidermis and serve as a source of water, binding free amino acids that contribute to skin hydration.[75, 109, 111, 112] In addition, matriptase-deficient animals also exhibit a defect in the formation of lamellar granules, specialized secretory vesicles that contain lipid material required for the formation of extracellular lipid lamellae within the cornified layer, and display impaired formation of epidermal tight junctions within the granular layer.[109, 113, 114] Those defects lead to some severe

forms of ichthyotic diseases, including lamellar and harlequin ichthyosis.[115-120] In conclusion, based on the mouse studies, a complete loss of matriptase activity in humans would be expected to be lethal.

Studies have shown that proteolytic activity of matriptase is strictly regulated during embryogenesis by a transmembrane Kunitz-type serine protease inhibitor, called hepatocyte growth factor activator inhibitor (HAI-1). HAI-1 is co-expressed with matriptase in embryonic and adult tissues through a complex with matriptase.[82, 121, 122] The identification of matriptase/HAI-1 complexes was first documented in human milk.[43] These complexes were also found in conditioned medium of cultured mammary epithelial cells, as well as in a number of cancer cell lines.[123]

1.5.4 Matriptase in epithelial cancers

Matriptase has consistent expression in many tumors of epithelial origin. It was first found in breast carcinoma[72, 124] and was later detected with high levels in a wide variety of other benign and malignant tumors of epithelial origin including prostate carcinoma,[125] ovarian cancer,[126] cervical cancer,[127] gastrointestinal cancers, kidney tumors,[75, 128] esophageal, oral squamous tumors,[129] head and neck squamous cell carcinoma, but it was not expressed in tumors of mesenchymal origin.[57, 75]

In many carcinomas, tumor progression is associated with a significant increase in matriptase mRNA and protein expression. Thus, in ovarian cancer cells, more than 5-fold overexpression of matriptase was detected, compared to normal ovarian tissues. The increased expression of matriptase correlated with the aggressiveness of the tumor, whereas it was downregulated in advanced-stage tumors.[126, 130] By contrast, in malignant tissue of the gastrointestinal tract, both the expression of mRNA matriptase and its cognate inhibitor HAI-1 were decreased in comparsion with the normal tissue.[131] In many cases, matriptase was more frequently expressed in stage I/II tumors (82%) than in more advanced stage III/IV tumors (55%). However, an increased matriptase/HAI-1 ratio was indicative of the poor clinical outcome of advanced-stage tumors, indicating that loss of matriptase inhibition may play a role in the late stages of the disease.[132] This output leads to the suggestion that an imbalance of the matriptase/HAI-1 ratio towards higher enzymatic activity contributes to tumor progression, including invasion and metastasis. Furthermore, a clinical study on breast cancer patients identified a correlation among expression of matriptase, HAI-1, the

putative matriptase substrate pro-HGF, and the cytoplasmic form of the HGF receptor/c-Met, suggestive of the pathophysiological relevance of co-expression of matriptase with a potential growth factor substrate.[133] Another study has shown that the activity of matriptase is also regulated by its glycosylation pattern. Matriptase was identified as a substrate for *N*-acetylglycosaminyltransferase V (GnT-V), which is an enzyme that catalyzes the β1-6 GlcNAc branching of *N*-glycans on the protein surface. The overexpression of GnT-V in gastric cancer cells leads to differences in the glycosylation pattern and therefore, to an increased matriptase activity due to an acquired resistance to its degradation, which contributes to malignant transformations.[134]

1.5.5 The role of matriptase in cartilage destruction in osteoarthritis (OA)

Recent studies highlighted the importance of serine proteases and MMPs in cartilage degradation and collagenolysis.[135] These proteases regulate the turnover of the ECM and of its major structural component type II collagen.[136] Therefore, these enzymes emerged as potential therapeutic targets.[137] Meanwhile, it was found that matriptase is overexpressed in OA cartilage disease and it might be an important mediator of catabolic processes through inducement of collagenolysis and activation of procollagenases. Studies have confirmed that matriptase activates PAR-2 and demonstrated that matriptase-dependent enhancement of collagenolysis from OA cartilage is blocked by PAR-2 inhibition.[138-140] PAR-2 activation in OA cartilage induces both MMP-1 and MMP-13 at the cell surface and this facilitates the pericellular collagenolysis within this focal region of the cartilage.[139] Furthermore, matriptase activity in OA cartilage leads to the induction of collagenolytic MMPs (MMP-1, MMP-3 and MMP-13) by the activation of their proforms. It was suggested that this process occurs via PAR-2 activation. Consequently, matriptase is considered as inducer of cartilage destruction in OA and becomes an attractive new therapeutic target for preventing pathologic cartilage breakdown and OA treatment.[141]

1.5.6 Natural matriptase inhibitors and their derivatives

HAI-1 is a potent endogenous inhibitor of the membrane-bound trypsin-like serine proteases matriptase, prostasin, HAT, TMPRSS13, and hepsin. It is a type I transmembrane protein with a cytoplasmic C-terminus that contains two extracellular

Kunitz-type inhibitor domains similar to BPTI (bovine pancreatic trypsin inhibitor, aprotinin, K_i = 10 nM for matriptase), which are separated by a low density lipoprotein receptor domain.[43, 142-144] It was shown recently that matriptase also forms a stable complex with the related kunitz-type inhibitor HAI-2.[145] Kunitz-type protease inhibitors bind to the active site of trypsin-like serine proteases forming relatively stable enzyme/inhibitor complexes and blocking the proteolytic activity of their target proteases.[146]

Wild-type ecotins are highly potent inhibitors of various serine poteases, isolated from the periplasm of *Escherichia coli*.[147] Ecotin and its double mutant (M84R/M85R) inhibit matriptase with K_i values of 782 pM and 9.8 pM, respectively. These optimised ecotin variants potentially inhibit the growth of prostatic carcinomas.[69, 148, 149]

SFTI-1 (sunflower trypsin inhibitor-1) is another natural matriptase inhibitor, which was originally isolated from sunflower seeds and found to be a potent trypsin inhibitor. It is a bicyclic 14-amino acid long peptide, containing a disulfide bridge and a head to tail cyclization. It exhibits strong inhibitory potency against matriptase with a K_i value of 0.92 nM (SFTI-1 structure **1**, Fig. 1.5,). SFTI-1 and several analogues have been synthesized by Roller's group. These bicyclic peptide analogues were used to investigate the structural basis of their inhibitory activity using molecular modeling.[150]

To improve the metabolic stability of the disulfide bridge in SFTI-1, both cysteine residues were replaced with allylglycine, followed by cyclization via ring closure metathesis. The reduced analogue **2** has a slightly decreased matriptase affinity (K_i = 2.5 nM). An alternative approach was the insertion of an additional methylene group between both sulphur atoms of the disulfide bridge resulting in the less active analogue **3** (K_i = 0.16 μM).[151]

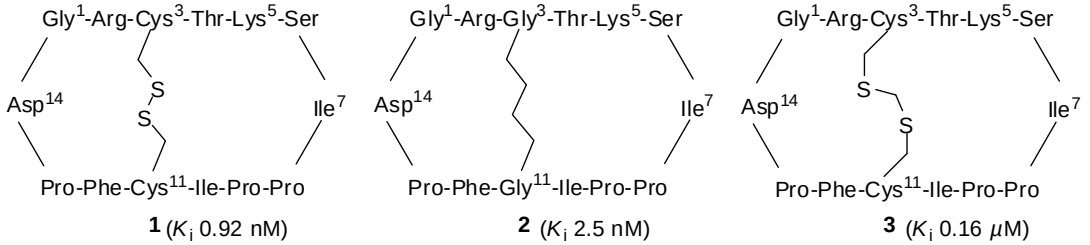


Fig. 1.5 Structures of SFTI-1 **1** and its analogues **2** and **3** including their K_i values against matriptase.[150, 151]

Recently, a crystal structure of the SFTI-1/matriptase complex was solved (pdb code: 3p8f). This complex revealed the binding of the Lys5 from the "reactive loop" of

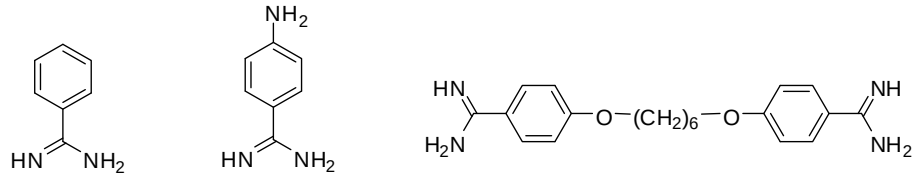
SFTI-1 in the S1 pocket of matriptase. It forms hydrogen bonds with both the Ser190 carbonyl oxygen and its hydroxyl side chain, as well as with an additional conserved water molecule. Surprisingly, no salt bridge to the Asp189 was found. The positively charged guanidinium group of Arg2 is directed towards the S3/4 site, forming cation-π interactions with the aromatic matriptase residues Phe97, Phe99 and Trp215.[76] This structure of the SFTI-1/matriptase complex provides a rational start point for designing new SFTI-1 derivatives with better potency and selectivity for matriptase and other proteases.

Human monoclonal antibodies (MAbs) were explored as matriptase inhibitor since matriptase is expressed on the cell surface, where large molecules can easily access the protease domain. As therapeutic reagents, human MAbs can be far less toxic and more selective than other types of inhibitors. Meanwhile several therapeutic antibodies, mainly directed towards growth factors or their receptors, have been approved as anti-cancer drugs. A phage display approach enabled the identification of a set of single chain variable region fragments (scFv) comprising the heavy- and light-chain variable regions (V_H and V_L), linked together by a peptide spacer.[152] Some of these chimeric scFv proteins, having a molecular weight of approximately 30 kDa, efficiently block the active site of matripaste with binding constants in the range from 50 pM to 130 nM. Some of these analogues were recently characterized as slow tight binding inhibitors of matriptase.[153] These antibodies constitute a new class of highly selective protease inhibitors that can be used to dissect the biological roles of proteolytic enzymes as well as to develop diagnostic and therapeutic reagents. The disadvantage of such antibodies, including their smaller fragments or naturally occurring oligopeptidic and protein-like protease inhibitors, is their relatively large molecular weight, which prohibits their oral bioavailability. To overcome this problem many groups have initiated a search for small molecule matriptase inhibitors.

1.5.7 Synthetic small molecule matriptase inhibitors

Simple benzamidines, such as **4** and **5** (Fig. 1.6, structure **4**, **5**), inhibit matriptase with K_i values of 390 and 90 μM, respectively. Their affinity was further enhanced by dimerization, whereas a sufficient linker length was required to achieve an improved potency. The bisbenzamidines **6**, containing a hexa methylene linker inhibit matriptase with a K_i value of 0.92 μM (Fig. 1.6, structure **6**).[154] However, such bisbenzamidines

possess poor specificity against other trypsin-like serine proteases, although some compounds have a slight selectivity for matriptase over thrombin. A crystal structure of the benzamidine/matriptase complex showed that the benzamidine binds into the S1 pocket and is sandwiched by the segments of Ser190-Gln192 and Trp215-Gly216 as expected.[76, 77]



4 (K_i 390 µM) **5** (K_i 90 µM) **6** (K_i 0.92 µM)

Fig. 1.6 Simple benzamidine derivatives and their inhibition constants for matriptase.[155]

Peptide phosphonates are irreversible inhibitors of various serine proteases and can be served for the design of the activity-based probes (ABPs).[156, 157] These ABPs can be used for labelling of proteases on the surface of cancer cells. Biotinylated peptide diphenyl phosphonate probes were recently developed as matriptase inhibitors. A crystal structure of one analogue in complex with matriptase has showed that the phosphorous atom is covalently bound to the active site Ser195 and that the benzamidine is placed in the S1 pocket. The positively charged amidine forms the typical double salt bridges to the negatively charged Asp189.[158]

A series of substrate analogue peptidomimetics, containing a C-terminal arginal residue such as CVS-3983 (**7**) showed a significant improved matriptase affinity. Compound **7** inhibits matriptase with a K_i value of 3.3 nM and has strongly reduced affinitity towards the related proteases factor Xa, plasmin, tPA, and uPA with inhibition constants > 1 μM.[159, 160]

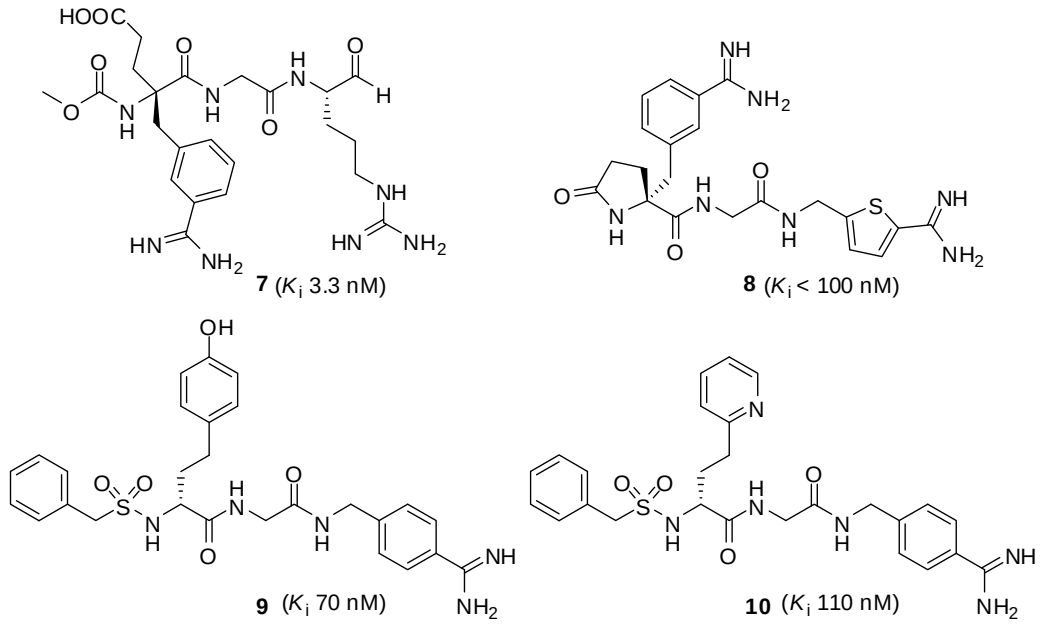


Fig. 1.7 Substrate analogue peptidomimetic matriptase inhibitors.[160, 161]

Although no structural information is available for matriptase in complex with CVS-3983 (**7**), it should be assumed that the arginal moiety forms a transition state analogue hemiacetal with Ser195, whereas the basic *m*-amidinophenyl side chain of the P3 amino acid is directed towards the aromatic S3/4 pocket. Most likely, cation/π-interactions contribute to inhibitor binding, similar to those described before for the binding of Arg39 in the BPTI-complex (Fig. 1.3).[77] Several analogues of CVS-3983 were synthesized to improve its inhibitory activity and affinity towards matriptase.[159] However, all these peptide aldehydes have limited value for clinical development due to their reactive P1 group. Therefore, the P1 arginal was replaced by decarboxylated Arg mimetics, known from the development of thrombin,[162, 163] factor Xa,[161] and factor VIIa inhibitors.[164] This has led to a series of new analogues, in which the P4-P2 segment was maintained or slightly modified. Several analogues have been disclosed in a patent application by Dendrion, e.g., the substituted pyroglutamyl compound **8** (Fig. 1.7) which inhibits matriptase with a K_i value < 100 nM.[165] During selectivity measurements with highly potent substrate analogue fXa inhibitors, several compounds with moderate matriptase affinity were found. For example, compounds **9** and **10** inhibit matriptase with K_i values of 70 and 110 nM, respectively (Fig. 1.7).[161]

Another type of matriptase inhibitors are sulfonylated 3-amidinophenylalanine derivatives. For instance, WX-UK1 (**11**, Fig. 1.8) inhibits matriptase with a moderate

K_i value of 0.37 μM, and was originally described as uPA inhibitor.[166] Compound **11** inhibits other trypsin-like serine proteases, such as thrombin and plasmin with comparable efficacy ($K_i \approx 0.5$ μM).[166] In preclinical studies, WX-UK1 effectively blocks metastasis formation and primary tumor growth.[167] The oral bioavailable hydroxyamidino prodrug of WX-UK1 (or mesupron **12**, Fig. 1.8)[168] is presently under clinical phase II development, and is used as a single agent or in combination with the chemotherapeutic 5-fluorouracil prodrug capecitabine for the treatment of patients with breast and pancreatic cancer (www.wilex.de).

Additional 3-amidinophenylalanine derivatives have been described as matriptase inhibitors and possess improved affinity and selectivity in case of the incorporation of a C-terminal guanidino group. For instance, the related bibasic derivative **13** (K_i value 57 nM) inhibits matriptase with at least 5-fold stronger than the analogue **11**.[78] The elongation of the C-terminal alkyl chain has not altered the potency of inhibitor **14** (K_i value 61 nM), whereas its cyclization resulted in inhibitor **15** (K_i = 14 nM) with enhanced matriptase affinity. An X-ray analysis of **15** in complex with the catalytic domain of matriptase (Fig. 1.9A) revealed a strong double hydrogen bond of the distal guanidino group to the carbonyl oxygen of Ile60, the first residue of the 60 insertion loop. The other interactions are similar to related complexes of arylsulfonylated tertiary amides of 3-amidinophenylalanine with trypsin, thrombin, or uPA.[78, 169, 170] The inhibitor adopts a compact Y-shaped conformation with the benzamidine segment placed in the S1 pocket forming a double salt bridge to Asp189, whereas the backbone of the 3-amidinophenylalanine makes antiparallel hydrogen bonds to Gly216. The conformation is further stabilized by a hydrogen bond between the sulfonyl oxygen and the amide moiety of Gly219.

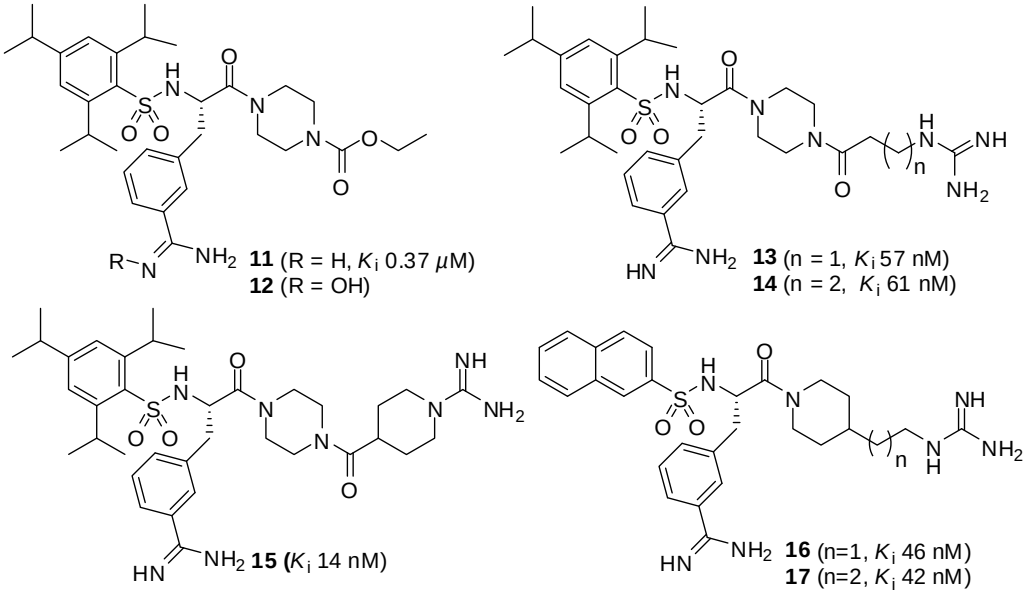


Fig. 1.8 3-Amidinophenylalanine-derived matriptase inhibitors.[78, 167-170]

Furthermore, an elimination of the C-terminal peptide bond provided compounds, such as **16** and **17** (Fig. 1.8) with K_i values of 46 and 42 nM, respectively. A similar overall conformation in X-ray studies with inhibitor **16** (Fig. 1.9B) as found for inhibitor **15** showed that the distal guanidinoethyl group is directed to a so called "cation cleft" formed by the initial part of the 60 insertion loop and the active site cleft of matriptase, which is lined by the carboxylates of Asp96 and Asp60b and the carbonyls of His 57 and Ile60. However, due to its truncated length the distal guanidinoethyl group forms only water mediated hydrogen bond to the side chain of Asp60b and does not interact with Ile60, as found in the complex with analogue **15**. In both complexes only loose contacts between the hydrophobic arylsulfonyl goups of the inhibitors and the matriptase S3/4 subsites were found. This is probably caused by the Arg60c side chain of a symmetry related matriptase molecule, which extends into this binding pocket under crystallization conditions and prevents a proper binding of the aryl sulfonyl group.

a)

b)

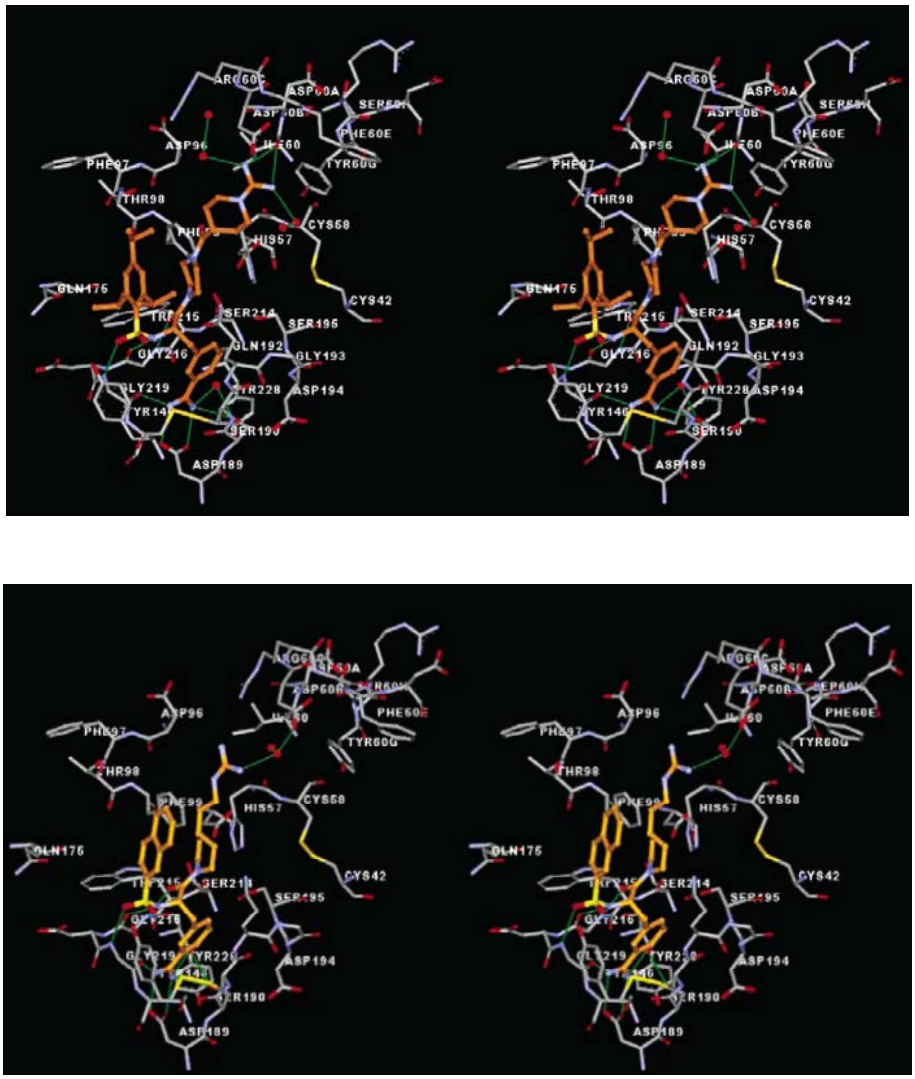
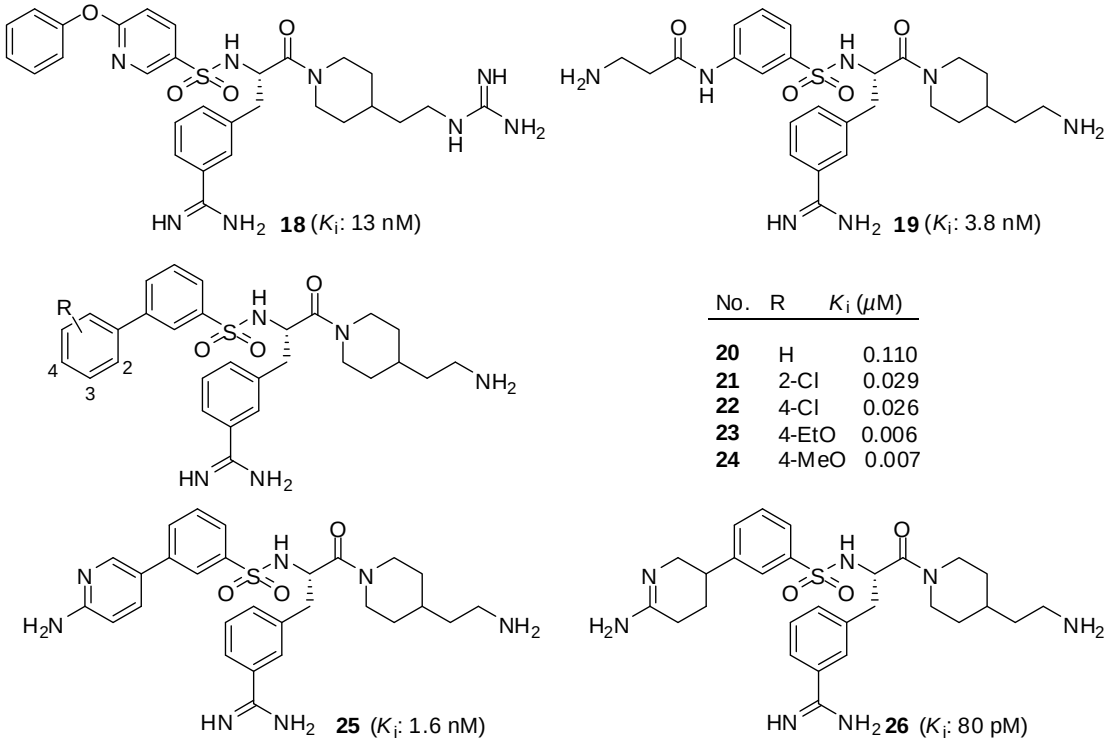


Fig. 1.9 Stereoview of inhibitors **15** (a, 2gv7) and **16** (b, 2gv6) bound to the active site of matriptase. Selected matriptase residues are labeled, and strong hydrogen bonds are shown in green. Only water molecules that are involved in strong hydrogen bonding are shown in red balls (figure adapted from Steinmetzer, 2006[78]).

Additional potent analogues of this inhibitor type were obtained by substitution of the phenylsulfonyl ring, e.g., inhibitor **18** (K_i value 13 nM, Fig. 1.10). It was also found that some tribasic 3-amidinophenylalanines inhibitors with an N-terminal of β-alanine connected via peptide bond to the *m*-position of the phenylsulfonyl group have an excellent selectivity for matriptase over related trypsin-like serine proteases. For instance, compound **19** inhibits matriptase with a K_i value of 3.8 nM, whereas the inhibition constants for thrombin, factor Xa, urokinase-type plasminogen activator, and plasmin are > 1 μM. The modeled complex of inhibitor **19** in matriptase revealed a binding of the β-alanyl group into the characteristic S3/4 site of the proteases. It was

assumed that the high potency of this inhibitor could be related to the formation of hydrogen bonds to carbonyl oxygen atoms of matriptase or cation-π-interactions between the protonated amino group and the aromatic residue (Trp215, Phe99, and Phe97) in this binding pocket.[78, 171]



No.	R	K_i (μM)
20	H	0.110
21	2-Cl	0.029
22	4-Cl	0.026
23	4-EtO	0.006
24	4-MeO	0.007

Fig. 1.10 Additional 3-amidinophenylalanine-derived matriptase inhibitors.[78, 171]

Tribasic inhibitors like compound **19** have a negligible oral bioavailability. Although several prodrug strategies are known for the amidino group as described for the thrombin inhibitors ximelagatran and dabigatran,[163, 172] no convenient prodrugs have been described for primary amino groups. Most of the known amino prodrugs are relatively stable to enzymatic conversion and therefore, less suitable in practice.[173, 174] Therefore, the C-terminal amino group or the N-terminal β-alanyl-amide moiety was replaced with uncharged residues in additional analogues.

In a first approach, various substituted biphenyl-3-sulfonyl groups were incorporated at the N-terminus.[171] Replacement of the N-terminal β-alanyl-amide moiety in inhibitor **19** by a non-substitued phenyl ring caused ≈ a 30-fold decrease in potency and reduced the selectivity. Analogue **20** inhibits matriptase and thrombin with K_i values of 0.11 and 0.056 μM, respectively. However, a single chlorine substitution, especially in para- and ortho-position of the terminal phenyl ring results in a slightly enhanced matriptase

affinity, as found for compounds **21** and **22**. An alkoxy substitution in different positions of this ring in the case of inhibitors **23** and **24**, also improved the inhibitory potency compared to the non-substituted analogues (Fig. 1.10). The most potent matriptase inhibitor is the tribasic analogue **26**, which inhibits matriptase with a K_i value of 80 pM and was obtained as a side product at the final hydrogenation of inhibitor **25**.

Additional matriptase inhibitors were prepared by a modification of the C-terminal of tertiary amide, where 2-aminoethylpiperidide was mainly replaced by neutral residues in a related series. Compound **28**, still containing the N-terminal β-alanine amide retained high potency and selectivity against matriptase (K_i value 6.3 nM) and was only 4-fold less active than inhibitor **27** (K_i value 1.8 nM). By contrast, compound **29** was relatively weak inhibitor (K_i value 53 nM).

In a further series, inhibitors with a C-terminal 4-piperidyl-butanoyl methylamide residue and an N-terminal biphenyl-3-sulfonyl residue were synthesized. Some of these analogues were found to be even more potent thrombin inhibitors like inhibitor **30**, which inhibits matriptase and thrombin with K_i values of 28 and 5.5 nM, respectivly. Analogue **31** maintains moderate affinity to matriptase with an inhibition constant of 87 nM, but shows only a weak potency against thrombin with a K_i value 2.05 μM. It seems that the more bulky *tert*-butyl substituted biphenyl group of compound **31** is not able to occupy the hydrophobic aryl binding site of thrombin, which is more open in case of matriptase. Inhibitor **32** (Fig. 1.11) is a dibasic analogue of inhibitor **26** containing an N-terminal 3-(6-amino-2,3,4,5-tetrahydropyridin-3-yl)benzenesulfonyl group, and found to be a very potent matriptase inhibitor with a K_i value of 0.43 nM.[175]

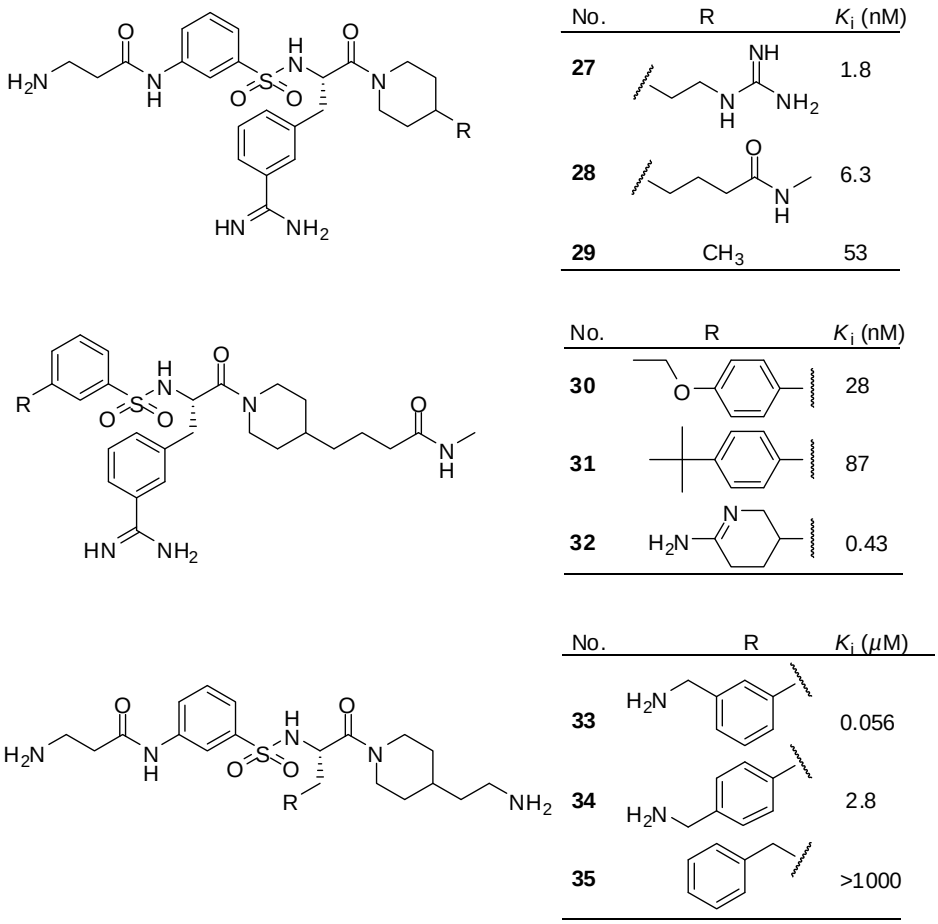


Fig. 1.11 Modifications of the C-terminal residue (**27**-**29**), the N-terminal biphenyl moiety (**30**-**32**) and the P1 residue (**33**-**35**).[171, 175]

The replacement of the 3-amidino group in P1 by an amino methylene residue in inhibitor **33** led to a significant decrease in the inhibitory potency. This can be explained by partial elimination of important hydrogen/salt bonds to Asp189, Gly219, or Ser190 at the bottom and both sides of the S1 pocket. The affinity was further reduced by incorporation of the aminomethyl group in the para-position (compound **34**, Fig. 1.11). The elimination of the amidino group in inhibitor **35**, containing homophenylalanine in P1 position, resulted in a dramatic more than 100,000-fold weaker matriptase inhibition compared to the analogue **19** (Fig. 1.10).[171]

2 Aim of the work

Most-likely, matriptase is involved in several physiological and pathophysiological functions. It has emerged as a potential target for the development of new anti-invasiveness drugs for the treatment of cancer. In addition, matriptase seems to be involved in osteoarthritis, atherosclerosis and in the activation of certain viral surface glycoproteins. Therefore, in the present work new matriptase inhibitors with improved properties should be developed using various approaches.

- A further optimization of tertiary amides of arylsulfonylated 3-amidinophenylalanine derivatives, which were previously decribed as highly potent inhibitors of matriptase, should be performed including modifications in the N-terminal biphenyl 3-sulfonyl group and C-terminal amide residue. A few additional analogues would be prepared by replacement of the 3-amidinophenylalanine with other P1 amino acids residues.
- A model of the binding mode of these new inhibitors in complex with matriptase should be established. Due to the insufficient amount of matriptase available, the easily accessible thrombin could be used as a kind of working horse for crystallization in complex with one of the most potent new inhibitors. The experimentally obtained coordinates of the thrombin/inhibitor complex should be superimposed with the known crystal structure of matriptase.
- Due to some similarity of the S3/4 pockets between matriptse and fXa, a new lead structure should be developed based on previously described fXa inhibitors containing a central D-phenylglycine residue. A 3-amidinobenzoyl residue should be incorporated as P1 residue, whereby substituted piperazines could be used to address the S3/4 binding pockets of matriptase.
- Due to the similarity of the S1 pocket of matriptase and uPA, new inhibitors should be derived from previously described antranilamide-containing uPA inhibitors.
- Substrate analogues containing 4-amidinobenzylamide as P1 residue are potent inhibitors of various trypsin-like serine proteases. Only a few analogues of this

type have been previously described as matriptase inhibitors with moderate K_i values > 50 nM. Therefore, new substrate analogue inhibitors with improved potency and selectivity should be developed.

- It was recently discovered that matriptase could be a potential activator of the surface glycoprotein hemagglutinin of the H9N2 influenza A virus subtype. Therefore, in collaboration with the group of Prof. Dr. Garten and Dr. Böttcher-Friebertshäuser, Institute of Virology, Philipps University Marburg, selected compounds should be tested as inhibitors of H9N2 influenza virus propagation.

3 Results and discussion

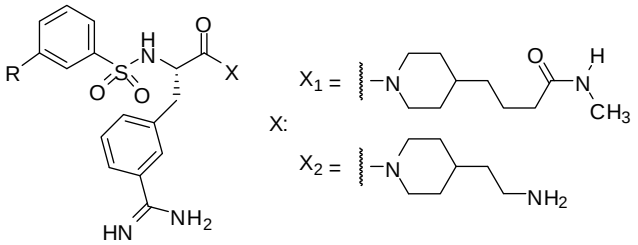
3.1 Development of new 3-amidinophenylalanine-derived inhibitors[1]

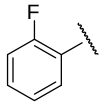
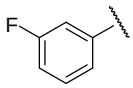
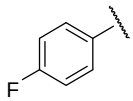
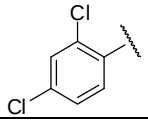
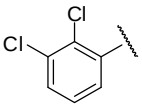
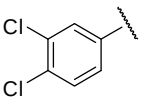
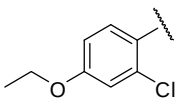
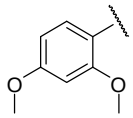
Piperidides and piperazines of sulfonylated 3-amidinophenylalanines were originally developed as inhibitors of the trypsin-like serine proteases thrombin and urokinase.[166, 176] Various analogues of this type were later identified as inhibitors of matriptase.[78] High potency and selectivity was found for tribasic 3-amidinophenylalanines, such as inhibitor **19** (Fig. 1.10, section 2.3),[171] which inhibits matriptase in the low nanomolar range and is a relatively poor thrombin inhibitor with a K_i value of 27 μM. To reduce their strong basicity associated with poor cell permeability, various substituted biphenyl-3-sulfonyl groups were incorporated at the N-terminus,[171] and the C-terminal 2-aminoethylpiperidide was replaced by uncharged residues in a related series.[175] However, both modifications reduced the selectivity of these analogues as matriptase inhibitors. For instance, the dibasic inhibitor **18** (Fig. 1.10, section 2.3) inhibits thrombin with a K_i value of 56 nM, while the monobasic analogue **30** is even a stronger thrombin inhibitor with an inhibition constant of 5.5 nM. To enhance the overall performance of these matriptase inhibitors, new analogues of this inhibitor type were prepared by further optimizing the N-terminal phenylsulfonyl and the C-terminal amide groups by incorporation of uncharged substituents.

In a first series, new analogues based on modifications of the terminal aryl ring at the biphenyl-3-sulfonyl residue were synthesized, whereas two different C-terminal amide groups known from previous studies were maintained (Table 3.1).[171, 175] Since it was known that a single chlorine (compounds **19** and **20**) or alkoxy (compounds **21** and **22**, section 2.3) substitution at different positions of this ring improved the inhibitory potency compared to the non-substituted analogue **18**,[171] various new disubstituted derivatives were synthesized **213-228** (Table 3.1).

[1] This part **3.1** of the dissertation is nearly identical to our recently published paper.[177]

Table 3.1: Inhibition of matriptase and related trypsin-like serine proteases by inhibitors of the general formula.



No.	X	R	K_i (μM)			
			matriptase	matriptase-2*	thrombin	fXa
213 MI-0403	X_1		0.12	3.0	0.002	1.1
221 MI-0433	X_2		0.023	2.1	0.04	0.81
214 MI-0404	X_1		0.13	10	0.006	3.05
222 MI-0431	X_2		0.052	3.2	0.04	3.2
215 MI-0405	X_1		0.12	4.4	0.001	0.55
223 MI-0434	X_2		0.033	3.2	0.005	1.12
216 MI-0406	X_1		0.013	0.35	0.001	0.032
224 MI-0432	X_2		0.002	0.11	0.02	0.05
217 MI-0440	X_1		0.051	0.75	0.004	0.26
225 MI-0441	X_2		0.011	0.21	0.020	0.58
218 MI-0442	X_1		0.074	1.6	0.003	0.23
226 MI-0443	X_2		0.018	0.58	0.03	0.37
219 MI-0412	X_1		0.049	1.3	0.03	0.18
227 MI-0413	X_2		0.013	0.40	0.40	0.17
220 MI-0438	X_1		0.008	0.77	0.001	0.06
228 MI-0439	X_2		0.001	0.31	0.03	0.11

*All K_i measurements of matriptase-2 provided in this thesis were performed in the group of Prof. Dr. Michael Gütschow (Rheinische Friedrich-Wilhelms-Universität Bonn). The details of the enzyme kinetic measurements with matriptase-2 have been described in recent publication.[177, 178]

In general, all dibasic compounds containing the C-terminal 2-aminoethylpiperidide are approximately 3-6 fold more potent matriptase inhibitors than their monobasic analogues, confirming previous studies. A strong matriptase inhibition was obtained for the 2′,4′-dichlorobiphenyl inhibitor **224** having a K_i value of 2 nM, whereas the other dichloro derivatives **225** and **226** (of matriptase K_i values of 0.011 and 0.018 μM, respectively) have a similar potency as found for the mono-substituted analogues. The

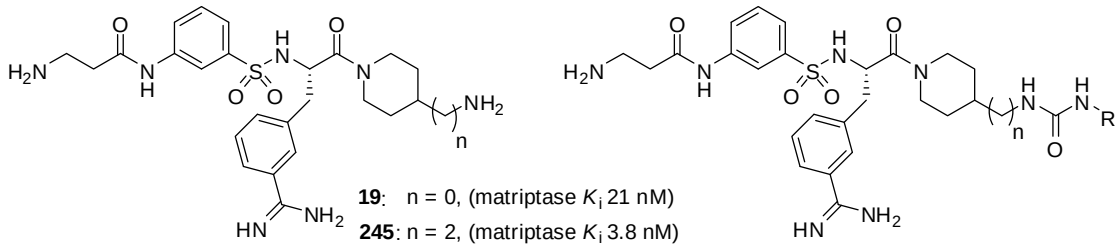
uncharged C-terminal *N*-methylamide containing analogues **216**-**218** inhibit matriptase with K_i values ≤ 0.074 μM. In addition, several fluorine-containing inhibitors were synthesized. The incorporation of fluorine atoms is a standard strategy to enhance affinity, metabolic stability and/or selectivity in pharmaceuticals.[179, 180] Due to its high electronegativity, the fluorine atom often interacts with electropositive regions of receptor sites and therefore strengthens protein-ligand binding.[181]

Among inhibitors **221**-**223**, the highest potency against matriptase was found for analogue **221** with a terminal 2-fluorophenyl ring with a K_i value 0.023 μM, while inhibitors **213**-**215** similarly inhibit matriptase with a K_i value 0.12 μM. However, several of these halogen-substituted analogues were found to be excellent thrombin inhibitors with K_i values < 6 nM, especially when they comprise the uncharged C-terminal N-methylamide group. This confirms results found by the Diederich group with other types of fluorine-substituted inhibitors that interact with the distal S3/4 pocket of thrombin.[182] The strongest matriptase inhibition with a K_i value of 1 nM was found for the dibasic 2,4-dimethoxy inhibitor **228**, which possesses an acceptable selectivity, as it is at least 20-fold less potent against the other tested proteases. Also in that case, its analogue **220** containing the uncharged C-terminal amide is a very potent thrombin inhibitor with a K_i value of 1 nM. Surprisingly, a relatively poor potency was found for inhibitor **227** (K_i 0.013 μM), which combines the single 4-ethoxy and 2-chloro substituents used previously. In general, all compounds are significantly less efficient factor Xa or matriptase-2 inhibitors. However, the strongest inhibition of both enzymes was also found for inhibitors **216** and **224**. It seems that their terminal 2,4-dichlorophenyl group contributes to the binding affinity to all tested trypsin-like proteases. As discussed before, most of the matriptase inhibitors summarized in Table 3.1 are also excellent thrombin inhibitors, which could induce bleeding complications, if such compounds would be used in animal experiments. A difference between both proteases is the structure of the characteristic 60-insertion loop in thrombin, formed by residues Tyr60A-Pro60B-Pro60C-Trp60D. It shields the binding pocket occupied by the C-terminal piperidide moiety in this inhibitor type as found in the crystal structure of inhibitor **224** with thrombin (Fig. 3.1a, section 3.1.1). By contrast, this binding region is relatively open in case of matriptase (Fig. 3.1b), since it contains a 60-insertion loop with completely different orientation compared to thrombin.

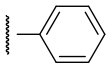
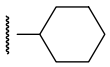
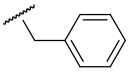
Therefore, a second inhibitor series was designed by incorporation of uncharged urea

structures in the C-terminal amide residue, which could be obtained by reaction of the respective amine precursors with various isocyanates (Scheme 3.2). We assumed that some of the rigid urea-containing inhibitors should have a reduced affinity to thrombin due to sterical repulsion of its 60 loop, whereby they maintain high affinity as matriptase inhibitors. To avoid the limited solubility due to the very hydrophobic character of these analogues, a *ß*-alanylamide substituted phenylsulfonyl group at the N-terminus in this series was initially incorporated, as known from inhibitor **19**.[78] The structures of the synthesized inhibitors, summarized in Table 3.2, differ in their C-terminal substituent at the urea moiety and in the distance between the piperidide and urea group.

Table 3.2: Inhibition of matriptase and related trypsin-like serine proteases by inhibitors of the general formula.



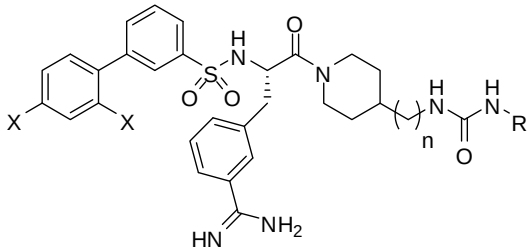
19: n = 0, (matriptase K_i 21 nM)
245: n = 2, (matriptase K_i 3.8 nM)

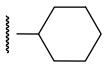
No.	n	R	K_i (μM)			
			matriptase	**matriptase-2**	**thrombin**	**fXa**
229 MI-0449	2	H	0.014	3.3	0.14	13.9
230 MI-0451	0		0.035	3.9	9.70	16.9
231 MI-0436	2	CH_3	0.007	1.2	0.04	7.07
232 MI-0450	0		0.056	1.3	10.8	14.1
233 MI-0437	2	CH_2-CH_3	0.012	0.46	0.13	4.79
234 MI-0452	0		0.039	2.4	15.31	8.89
235 MI-0444	2		0.011	1.9	0.44	2.61
236 MI-0454	0		0.014	1.3	9.25	3.41
237 MI-0445	2		0.023	1.8	0.47	6.45
238 MI-0453	0		0.008	0.79	35.9	1.2
243 MI-0446	2		0.017	1.3	0.23	5.52
244 MI-0455	0		0.026	1.1	7.84	2.45

Within the series containing a piperidylethyl-urea portion, the methyl compound **231** has the highest potency against matriptase with a K_i value of 7 nM, whereas its shorter analogue **232** is approximately 8-fold less active. All other analogues have reduced affinity, but still inhibit matriptase with inhibition constants < 25 nM. By contrast, in the series with the shorter piperidyl-urea portion, the cyclohexyl derivative **238** is the most potent matriptase inhibitor; possessing an inhibition constant of 8 nM and shows an excellent selectivity with respect to thrombin. However, its longer analogue **237** inhibits matriptase only with a K_i value 23 nM. The rigid cyclohexyl-urea group of inhibitor **238** seems to prevent a proper binding below the 60 insertion loop of thrombin. An additional reason for the reduced thrombin affinity in this series is most likely caused by the presence of the N-terminal *β*-alanylamide residue, because the distal S3/4 binding pocket of thrombin is less suited to accommodate charged basic groups. All analogues summarized in Table 3.2 show poor affinity to factor Xa and matriptase-2, only two compounds (**233** and **238**) inhibit matriptase-2 with K_i values < 1 μM.

In a third series (Table 3.3), monobasic inhibitors were obtained by replacement of the N-terminal *β*-alanyl amide moiety of the most potent urea inhibitors **231** and **238** with the 2,4-dimethoxyphenyl or 2,4-dichlorophenyl group, which were used in the most potent biphenyl-3-sulfonyl containing inhibitors **224** and **228**.

Table 3.3: Inhibition of matriptase and related trypsin-like serine proteases by inhibitors of the general formula.



No.	X	n	R	K_i (μM)					
				matriptase	matriptase-2	thrombin	fXa	uPA	HAT*
239 MI-0460	CH_3O	2	CH_3	0.0018	0.18	0.0008	0.051	4.4	0.11
240 MI-0461	Cl	2		0.0009	0.073	0.0002	0.029	3.4	0.17
241 MI-0462	CH_3O	0		0.0027	1.42	1.23	0.011	18.5	0.83
242 MI-0463	Cl	0		0.0051	0.94	0.63	0.02	18.1	2.7

*Measurements of the K_i values with HAT were performed by Daniela Meyer, as described previously.[183]

As expected, all four analogues have a slightly higher matriptase affinity compared to the β-alanyl series, whereas they possess strong differences in their thrombin inhibition. The piperidylethyl-urea derivatives **239** and **240** inhibit thrombin with K_i values < 1 nM, whereas both piperidyl-urea inhibitors substituted by a terminal cyclohexyl group (**241**, **242**) have a more than 500-fold reduced thrombin affinity. The X-ray structure of inhibitor **224** in complex with thrombin reveals that its C-terminal aminoethyl group is long enough to slip out of the 60-loop (Fig. 3.1a), which enables its further modification without sterical hindrance. This seems to be impossible in the case of the shorter aminopiperidyl inhibitors **241** and **242**. Their modification by sterically demanding urea structures leads to poor thrombin affinity but is well tolerated by matriptase, possessing an open prime site. This tendency is confirmed by the K_i values summarized in Table 3.2. Inhibitors **239**-**242** were tested also against the related enzymes uPA and HAT, whereby HAT is also a TTSP that belongs to the HAT/DESC subfamily. However, all compounds were relatively weak inhibitors of these proteases.

3.1.1 Modeled complex of inhibitor 224 in matriptase

Based on previously solved X-ray structures of matriptase in complex with closely related 3-amidinophenylalanine derivatives possessing N-terminal 2-naphthylsulfonyl- or 2,4,6-triisopropylphenylsulfonyl-groups,[78] we assumed that the terminal 2,4-dichloro-substituted phenyl ring of inhbibitor **224** binds to the distal S3/4 binding pocket of matriptase above the side chain of Trp215, which is covered by Phe99 at the right side, Gln175 at the left and Phe97 on the top. To confirm this binding mode we were interested to obtain a crystal structure with these biphenyl-3-sulfonyl-3-amidinohenylalanine-derived inhibitors. Due to the insufficient amount of matriptase available we used the easily accessible thrombin as a form of working horse for crystallization in complex with inhibitor **224** (Fig 3.1), which inhibits thrombin with a K_i value of 20 nM. The determined binding mode is shown in Figure 3.1a, which confirms that the biphenyl moiety is directed towards the distal S3/4 binding pocket of this related trypsin-like serine protease. The para-chlorine substituent is located 3.5 Å above the indole portion of Trp215 suggesting a halogen-π interaction[184] and in a distance of 3.2 Å away from the carbonyl oxygen of Asn98, probably making a polarized halogen-carbonyl contact.[185] The ortho-chlorine is involved in a van-der-Waals contact to the γ-methylene group of Glu217 (3.7 Å). All other interactions, the

binding of the amidino group to Asp189 and Gly219, the H-bond of one sulfonyl oxygen to the NH of Gly219, as well as the antiparallel β-sheet interaction between the 3-amidinophenylalanine backbone to Gly216 are identical as known from other thrombin structures with related inhibitors.[186] This complex was superimposed by an rmsd fit of the C_α-atomic coordinates onto a previously solved crystal structure of matriptase inhibited by a 2-naphthylsulfonylated 3-amidinohenylalanine-derived inhibitor (Fig 3.1b).[78] Based on this model, the N-terminal biphenyl group of inhibitor **224** also fits well into the S3/4 pocket of matriptase making a close edge to face contact to the aromatic side chain of its characteristic Phe99 (≈ 3.5 Å), whereas all characteristic H-bonds of the P1 3-amidinophenylalanine and one sulfonyl oxygen were maintained (Fig. 3.1c). It might be possible that in case of the matriptase complex an additional halogen-π-interaction between the para-chlorine substituent and the adjacent phenyl ring of Phe99 (≈ 3.7 Å) is established, which contributes to the improved binding of inhibitor **224**. Such contacts are known from many other examples.[184] Calculations of the electrostatic potential performed at complexes of factor Xa or thrombin with inhibitors containing chloroaromatic P1 groups that interact with Tyr228 at the bottom of the S1 pocket reveal a slightly positive charge located at the tip of an aromatic chlorine atom, which interacts with π-electron systems of the neighbouring aromatic amino acid.[187]

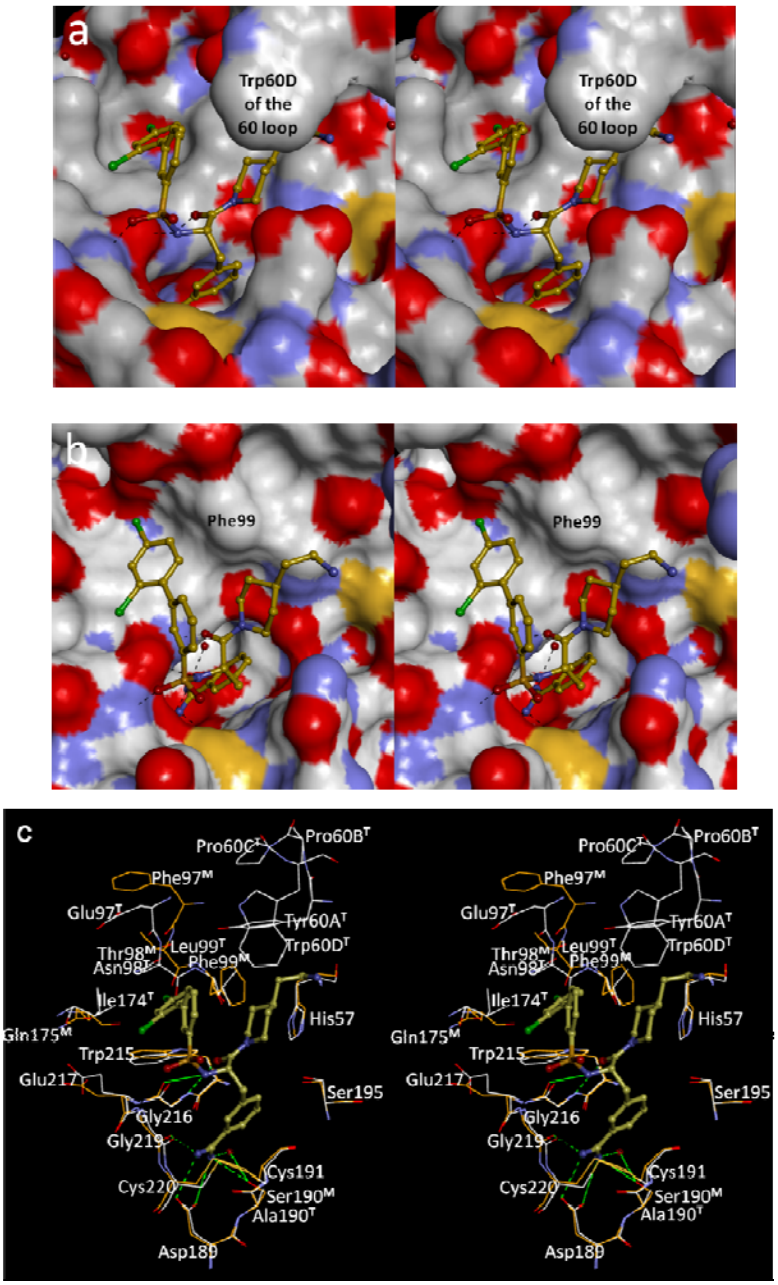


Fig. 3.1 a) Stereo view of the thrombin/**224** complex (4e7r.pdb). Thrombin is shown with its solvent-accessible surface colored by atom type (carbon in grey, oxygen in red, nitrogen in blue, sulfur in yellow) and the inhibitor in sticks. The C-terminal piperidide of inhibitor **224** is located below the characteristic 60 insertion loop of thrombin, whereby its terminal amino group sticks out from this loop. b) Model of inhibitor **224** in matriptase, shown with its surface colored by atom type. This stereo figure was obtained by superimposing the thrombin/**224** complex with the matriptase coordinates taken from the crystal structure with the pdb-code 2gv6,[78] followed by deletion of thrombin. Compared to thrombin, matriptase has a relatively open S1′ site and possesses a Phe in position 99, which is Leu in thrombin. c) Stereo view of thrombin (white carbons) in complex with **224** superimposed with matriptase (orange carbons). Hydrogen bonds formed between **224** and matriptase are shown as green lines, which match nearly perfectly with those found in thrombin/**224** complex. Two additional hydrogen bonds are formed from the side chain of Ser190 in matriptase to the amidino group of **224** and to a conserved water molecule at the bottom of the S1 pocket, which is Ala190 in thrombin. For clarity, only some important residues of both enzymes in the active site are shown and labeled. Identical residues in both enzymes are labeled by the common name, specific matriptase and thrombin residues are indicated by an additional M or T, respectively.

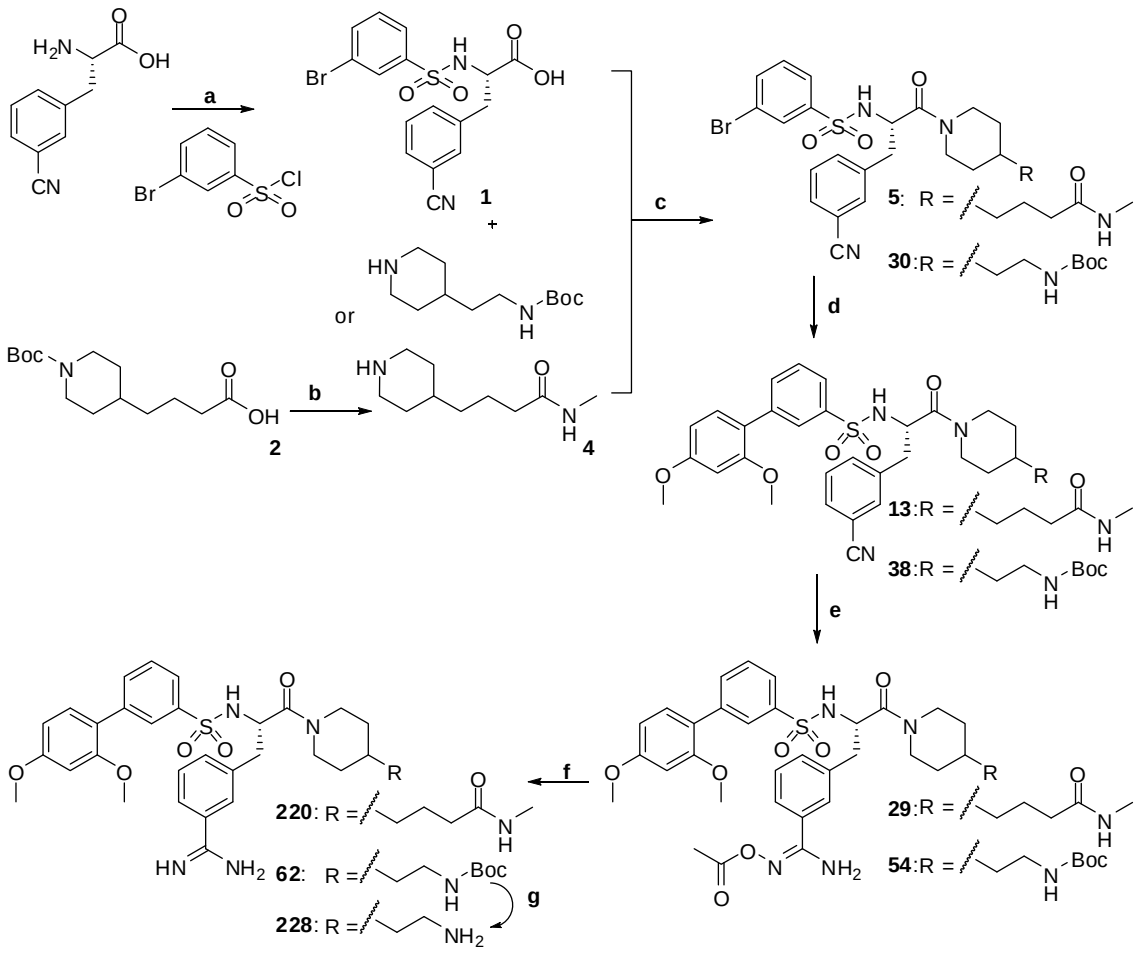
3.1.2 Synthesis of the 3-amidinophenylalanine-derived inhibitors

The synthesis of the inhibitors shown in Table 3.1 was performed according to previously described methods[78, 171, 175] using intermediates **5** and **30**,[78] which were prepared from the appropriate sulfonyl-protected 3-cyanophenylalanines performing the following steps in Scheme 3.1.

The C-terminal residue, *N*-methyl-4-(piperidin-4-yl)butanamide was prepared from a commercially available 4-(piperidin-4-yl)butanoic acid, which was Boc-protected, converted into its methylamide by treating with methylamine × HCl and HBTU as a coupling reagent in the presence of DIPEA in to obtain compound **3**, followed by cleavage of the Boc-group with 1 N HCl in HOAc providing intermediate **4** (Scheme 3.1). Intermediate **1** was obtained by coupling of 3-bromophenylsulfonyl chloride with 3-cyanophenylalanine under Schotten-Baumann conditions,[78] followed by coupling with the respective piperidines, *N*-methyl-4-(piperidin-4-yl)butanamide (intermediate **4**) to obtain compound **5**, or with *tert*-butyl 2-(piperidin-4-yl)ethylcarbamate[2] to obtain intermediate **30**, using PyBOP/DIPEA.

Intermediates **5** and **30** were treated with various arylboronic acids according to a Suzuki coupling protocol. For instance, 2,4-dimethoxyphenylboronic acid was used for the preparation of inhibitors **220** and **228**, using approximately 1 mol% $Pd(OAc)_2$ and 2 mol% SPhos in toluene in the presence of Cs_2CO_3 solution.[78, 171] The conversion of the nitrile into an amidine was performed according to the method of Judkins.[188] The nitrile in intermediates **13** and **38** were treated with hydroxylamine x HCl and DIPEA in ethanol, followed by treatment with acetic anhydride (e.g., intermediate **29** and **54**). These acetylhydroxyamidine compounds were hydrogenated using (10% Pd/C) as a catalyst (Scheme 3.1). In case of chlorine- and flourine-containing derivatives, this step was performed with zinc dust in 90% HOAc to avoid a partial removal of the aromatic halogen atoms during hydrogenolysis. Intermediates containing a 2-(piperidin-4-yl)-Boc-ethylamine residue were deprotected by treating with 90% trifluoroacetic acid to obtain the related inhibitors. The final inhibitors were purified by preparative HPLC, followed by lyophilization from 80% *tert*-butanol.

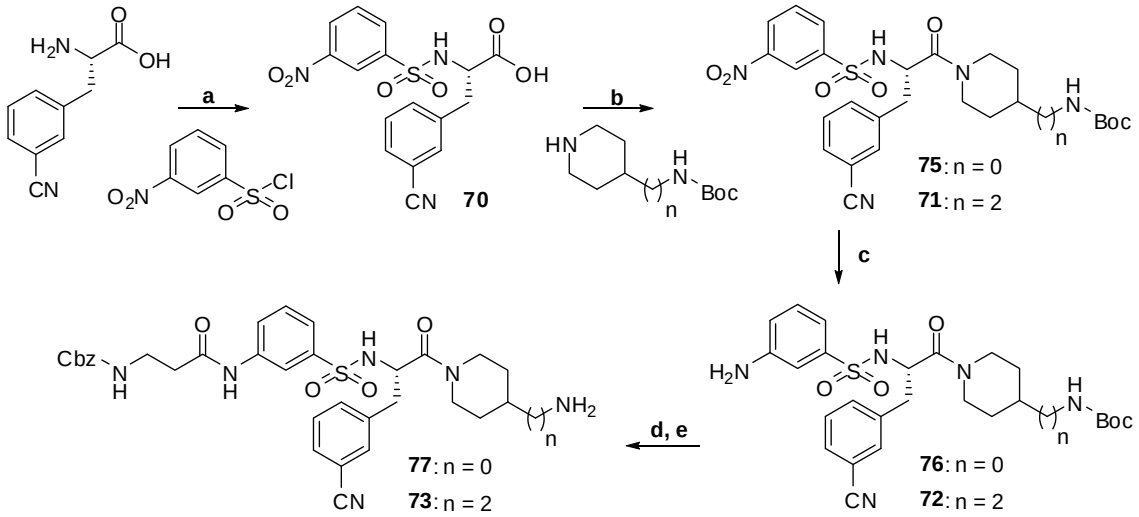
[2] This compound was available in our group from previous work.



Scheme 3.1 Synthesis of inhibitors **220** and **228**. Reagents and conditions: (a) 2.1 equiv DIPEA, 1.1 equiv sulfonyl chloride in dioxane and water (2:1, v/v) at 0 °C and at RT overnight; (b) i: 1 equiv intermediate **2**, 10 equiv methylamine × HCl, 1.5 equiv HBTU and 4.5 equiv DIPEA in EtOAc at 40 °C for 5 h; ii: intermediate **3** dissloved in 1 N HCl in HOAc, 1h; (c) 1.05 equiv PyBOP and 3 equiv DIPEA in DMF, 30 min at 0 °C, and 2 h at RT; (d) 1.5 equiv 2,4-dimethoxyphenylboronic acid, 1 mol% $Pd(OAc)_2$ and 2 mol% *S*-Phos in toluene containing 3 equiv 2 M Cs_2CO_3 solution, 3-4 h reflux; (e) i: 3 equiv hydroxylamine x HCl and 3 equiv DIPEA, 4 h reflux in ethanol, and stirring overnight at RT; ii: 3 equiv Ac_2O in HOAc, 30 min at RT; (f) H_2 and Pd/C as catalyst in 90% HOAc, stirring overnight at RT (in case of halogen-containing derivatives this step was performed with zinc dust in 90% HOAc to avoid a partial removal of the aromatic halogens during hydrogenolysis); (g) 90% TFA, at RT 1 h.

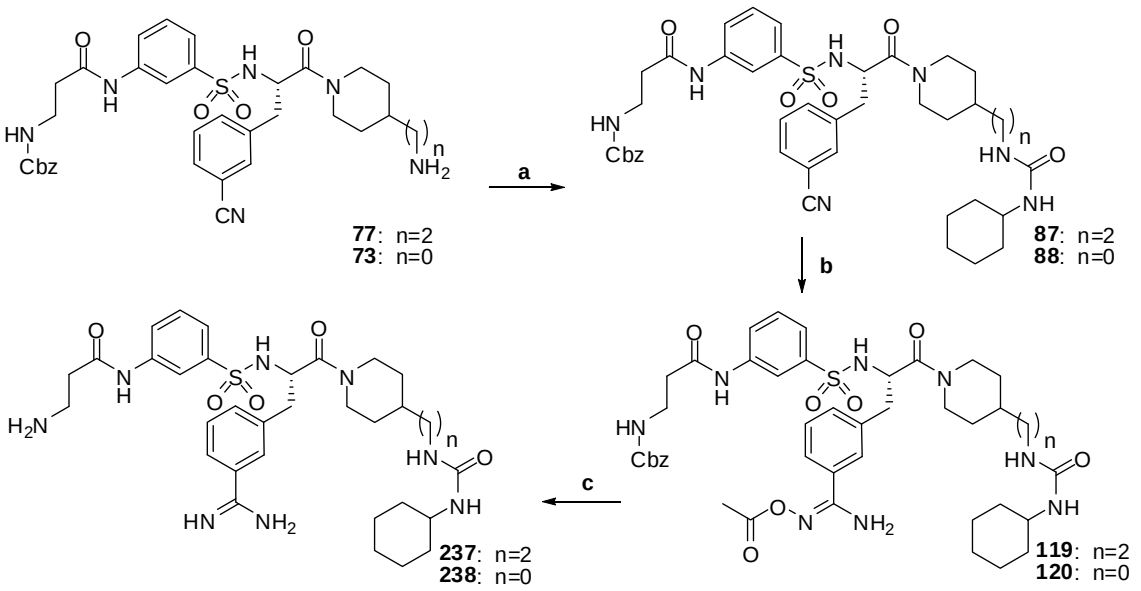
The inhibitors summarized in Table 3.2, were prepared from compounds **73** and **77**, which were synthesized according to Scheme 3.2 as previously described.[78] Intermediate **70** was obtained from coupling of 3-nitrophenylsulfonyl chloride with 3-cyanophenylalanine under Schotten-Baumann conditions, followed by coupling with *tert*-butyl 2-(piperidin-4-yl)ethylcarbamate or with commercially available *tert*-butyl piperidin-4-ylcarbamate × HCl using PyBOP/DIPEA to obtain intermediates **71** and **75**, respectively. The nitro group was reduced using zinc dust in 90% HOAc providing compounds **72** and **76**. These intermediates were coupled with Cbz-*β*-alanine using the

mixed anhydride procedure, followed by Boc-deprotection resulted in compounds **73** and **77**.



Scheme 3.2 Synthesis of intermediates **73** and **77.** Reagents and conditions: (a) 1.1 ml 1N NaOH, 1.1 equiv sulfonyl chloride in ACN and water (2:1, v/v) at 0 °C and at RT overnight; (b) 1.1 equiv *tert*-butyl 2-(piperidin-4-yl)ethylcarbamate or 1.1 equiv *tert*-butyl piperidin-4-ylcarbamate × HCl, 1.05 equiv PyBOP and 3 equiv DIPEA in DMF, 30 min at 0 °C, and 2 h at RT; (c) zinc dust in 90% HOAc 2 h at RT; (d) 1 equiv Cbz-β-alanine, 1 equiv isobutyl chloroformate and 1 equiv NMM, 10 min at -15 °C in DMF; 1 equiv intermediate **72** or **76**, 1 equiv NMM stirring 1 h at -15 °C and overnight at RT; (e) 90% TFA, at RT 1 h.

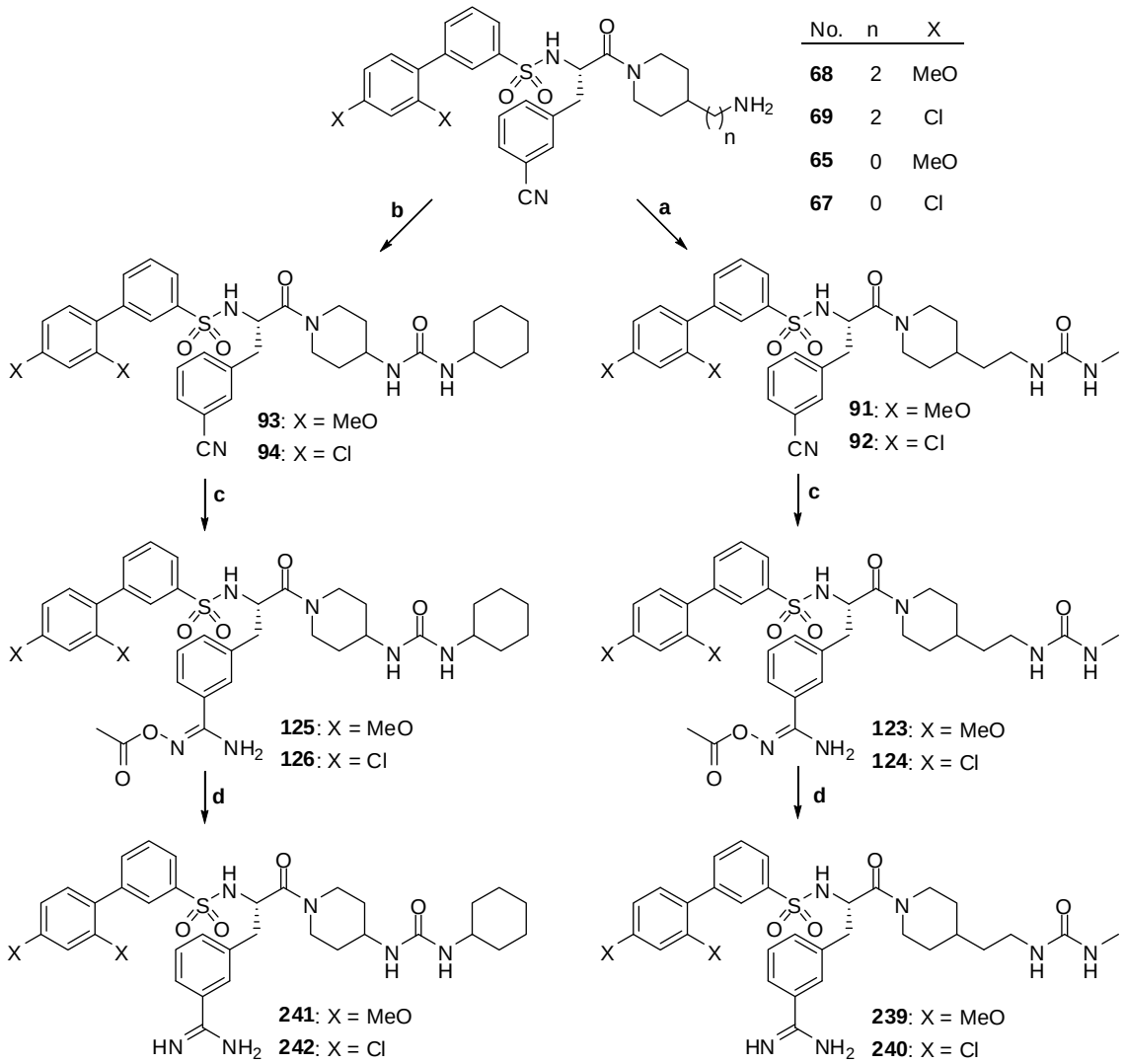
For most inhibitors summerized in Table 3.2, 1 equiv of the intermediates **77** or **73** was treated with 1.5 equiv of the appropriate isocyanates,[189, 190] e.g., cyclohexyl isocyanate for inhibitors **237** and **238**, and 2 equiv TEA in DCM according to Scheme 3.3. The nitrile containing compounds **87** and **88** were converted into acetylhydroxyamidine to obtain intermediates **119** and **120**, followed by hydrogenation using the previous described procedures providing the final inhibitors **237** and **238** as described in Scheme 3.1. In case of benzyl-containing intermediates **127** and **128**, the conversion of acetylhydroxyamidine into amidine was performed with zinc dust in 90% HOAc to avoid removal of the benzyl group during hydrogenolysis, followed by the final cleavage of the Cbz group with 32% HBr in HOAc to obtain inhibitors **243** and **244**.



Scheme 3.3 Synthesis of inhibitors **237** and **238**. Reagents and conditions: (a) 1.5 equiv cyclohexyl isocyanate and 2 equiv TEA in DCM, 3 h at RT; (b) i: 3 equiv hydroxylamine x HCl and 3 equiv DIPEA, 4 h reflux in ethanol, and stirring overnight at RT; ii: 3 equiv Ac_2O in HOAc, 30 min at RT; (c): H_2 and Pd/C as catalyst in 90% HOAc and stirring overnight at RT (in case of the benzylureas **127** and **128** this step was performed with zinc dust in 90% HOAc to avoid removal of the benzyl group during hydrogenolysis, followed by cleavage of the Cbz group with 32% HBr in HOAc).

The unsubstituted urea moiety of inhibitors **229** and **230** was synthesized by reaction of the amine intermediate with *N*-methyl-*N*-nitrosourea in DCM,[191] whereas the methylureas **231** and **232** were prepared by treatment of the amine with *N*-succinimidyl-*N*-methylcarbamate to avoid the use of methylisocyanate.[192]

Inhibitors **239**-**242** (Table 3.3) were prepared from compound **30** (Scheme 3.1), or its analogue **63** possessing the shorter C-terminal Boc-aminopiperidide residue. These compounds were coupled with the appropriate 2,4-dichloro-phenylboronic acid or 2,4-dimethoxy-phenylboronic acid using a Suzuki coupling procedure (conditions according to Scheme 3.1), followed by cleavage of the Boc group using 90% trifluoroacetic acid. The intermediates **68** and **69** (Scheme 3.4) were treated with 1.5 equiv *N*-succinimidyl-*N*-methylcarbamate[192] and 2 equiv TEA in DCM to obtain compounds **91** and **92**. However, the urea moiety of intermediates **93** and **94** was obtained by treating compounds **65** and **67** with cyclohexyl isocyanate. The nitrile was converted into an amidino group as described previously.



Scheme 3.4 Synthesis of inhibitors **239-242**. Reagents and conditions: (a) 1.5 equiv *N*-succinimidyl-*N*-methylcarbamate and 2 equiv TEA in DCM, 3 h at RT; (b) 1.5 equiv cyclohexyl isocyanate and 2 equiv TEA in DCM, 3 h at RT; (c) i: 3 equiv hydroxylamine x HCl and 3 equiv DIPEA, 4 h reflux in ethanol, and stirring overnight at RT; ii: 3 equiv Ac_2O in HOAc, 30 min at RT; (d) zinc dust in 90% HOAc, 3 h at RT.

3.1.3 Inhibition of H9N2 influenza virus propagation by matriptase inhibitors[3]

The infectivity and replication of influenza viruses depends on the proper cleavage of its surface glycoprotein hemagglutinin (HA) that mediates binding to sialic acid-containing cell surface receptors and fusion of the viral envelope with the endosomal membrane. HA is synthesized as a precursor protein HA0 and needs to be cleaved by a host cell protease into the subunits HA1 and HA2 to gain its fusion capacity.[193, 194]

[3] These studies on the inhibition of H9N2 virus propagation by matriptase inhibitors were performed in the group of Dr. Eva Böttcher-Friebertshäuser and Prof. Dr. Wolfgang Garten in the Institute of Virology, Philipps University Marburg.

In cell culture experiments, it was demonstrated that some serine proteases, like TMPRSS2 and HAT, activate the HA of the H1N1 and H3N2 influenza strains containing a monobasic cleavage site.[195] By contrast, matriptase was not able to cleave these hemagglutinins of the presently circulating human pathogen influenza strains. However, matriptase was recently discovered to be a potent activator of the HA from H9N2 influenza A virus subtype (Joanna Baron and Dr. Eva Friebertshäuser, unpublished data). The used subtype was first isolated from a quail in China (Province Shantou) in 2000. These H9 influenza strains are considered to possess the potency to be transformed into a new panademic virus strains.[196] Therefore, the relatively selective and potent matriptase inhibitors **19**, **224**, **241,** and **242** were examined for their inhibitory effect on the propagation of H9N2 influenza virus in MDCK II cells[4] (Fig. 3.2). These cells strongly express matriptase, which is considered to be the revelant protease that specifically activates the HA of influenza virus subtype H9N2 at the cleavage site sequence R-S-S-R↓.

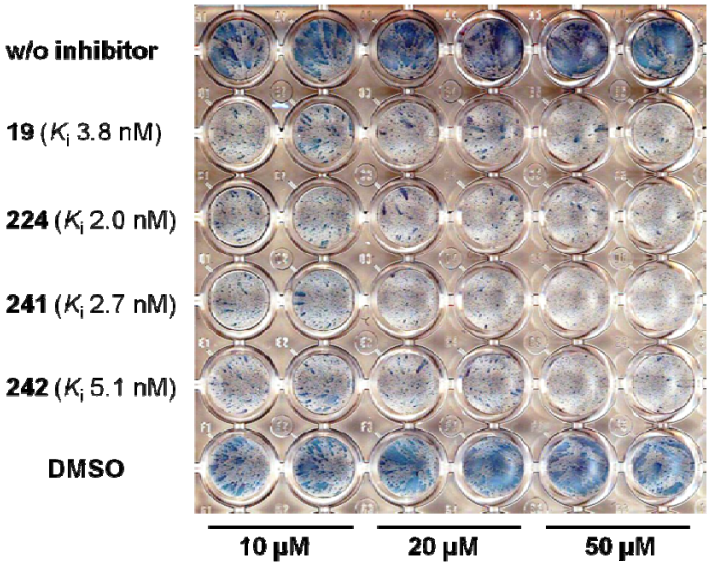


Fig. 3.2 Inhibition of H9N2 influenza virus propagation by treatment with matriptase inhibitors in MDCK-II-matriptase cells. MDCK II cells were infected with the virus A/quail/Shantou/782/00 (H9N2) and incubated with the respective inhibitors at 37° C for 24 h in an incubator to allow virus propagation. After 24 h the infected cells and comet-like spread of infection were fixed with 4% paraformaldehyde in minimum essential medium. These cells were incubated with specific first antibodies against the viral nucleoprotein (NP) of H9N2, followed by conjugation with horse radish peroxidase (HRP) secondary antibodies. Infected cells were immunostained using the peroxidase substrate TrueBlue (KPL Inc.) as described previously.[50, 195, 197]

[4] Madin–Darby canine kidney II (MDCK-II) cells are a kidney epithelial cell line, routinely used for the culturing of influenza viruses. It was proven by quantitative PCR that these cells express a relative high amount of mRNA for matriptase. These cells allow an efficient H9N2 virus propagation without exogenous trypsin treatment.

As expected, within 24 hours a multicycles replication of viruses is observed as blue comet-like spots in control and DMSO-treated cells in the absence of inhibitors. It is assumed that endogenous expressed matriptase is able to activate the newly formed H9. For the purpose of evaluation, the comet-like spread of the virus in untreated or DMSO-treated cells was compared to a concentration-dependent reduced spread in inhibitor-treated cells. All selected inhibitors and the reference compound **19** strongly suppress multicycle virus replication at a concentration of 50 μM (Fig. 3.2). The strongest inhibition was found for the cyclohexylurea derivative **241**, which is more effective than reference **19** containing an N-terminal β-alanine residue.[78] Inhibitor **241** completely inhibits the virus replication at concentration 50 μM, whereby it has also a strong inhibitory effect at reduced concentrations of 10 and 20 μM. It should be noted that compound **241** is also a very effective matriptase inhibitor with a K_i value of 2.7 nM. The other three compounds inhibited the virus spread slightly less, although they possess very similar K_i values. These results demonstrate that the tested matriptase inhibitors are effective in suppressing the replication of H9N2 influenza viruses in a matriptase-expressing MDCK II cell model.

3.2 Replacement of the P1 3-amidinophenylalanine

A few additional analogues were initially synthesized by replacement of the 3-amidinophenylalanine (Table 3.4), because various other amino acids have been used in P1 position of inhibitors prepared for other trypsin-like serine proteases.

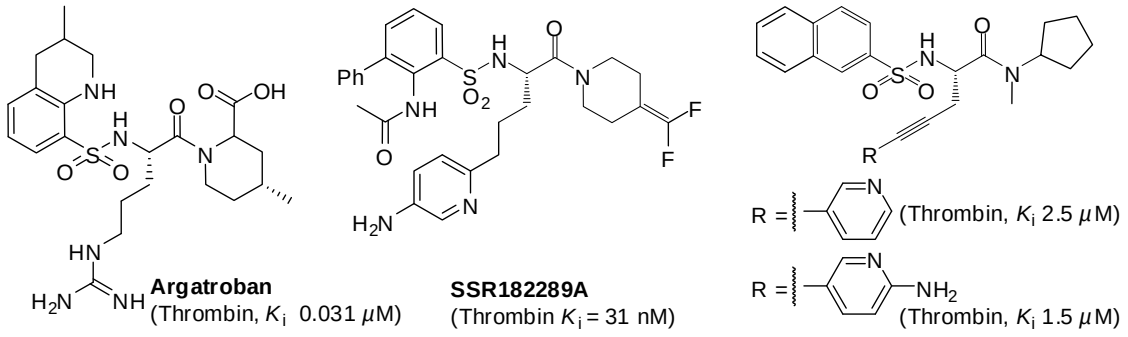


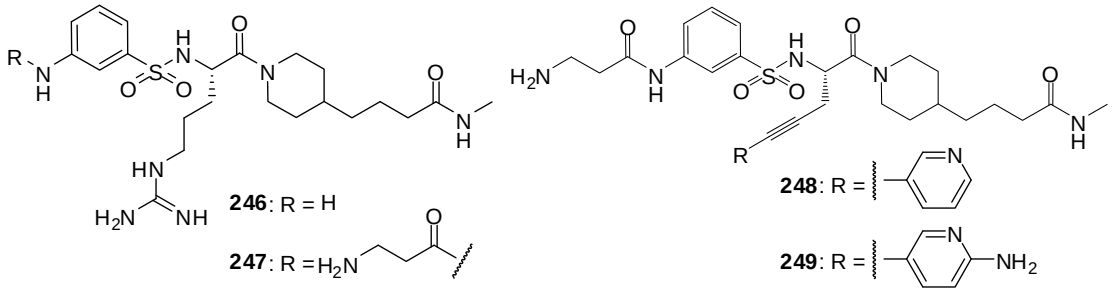
Fig. 3.3 Structures of known thrombin inhibitors containing different P1 residues.[198-200]

For instance, an arginine was introduced as P1 residue in the clinically used thrombin inhibitor Argatroban.[201] However, the incorporation of a P1-arginine in inhibitor **247**, which is a direct analogue of compound **28** (K_i value for matriptase 6.3 nM, Fig. 1.11),

led to an approximately 100-fold reduction in potency with a K_i value of 0.7 μM. The precursor **246**, missing the N-terminal β-alanyl residue, is even more than a 1000-fold weaker matriptase inhibitor.

Inhibitors **248** and **249** containing pyridylpropargylglycine derivatives as P1 residue were synthesized in analogy to previously described thrombin inhibitors[200] (Fig. 3.3), e.g., compound **249** has some similarity to the previously described thrombin inhibitor.[199] The elimination of the strongly basic amidino group in these compounds results in more than a 10000-fold weaker matriptase inhibition, most likely due to complete or partial elimination of important hydrogen/salt bonds to Asp189, Gly219, and Ser190 at the bottom and at both sides of the S1 pocket. In general, all these analogues are very poor matriptase inhibitors; therefore, no further analogues were prepared.

Table 3.4: Inhibition of matriptase and related trypsin-like serine proteases by inhibitors of the formula.

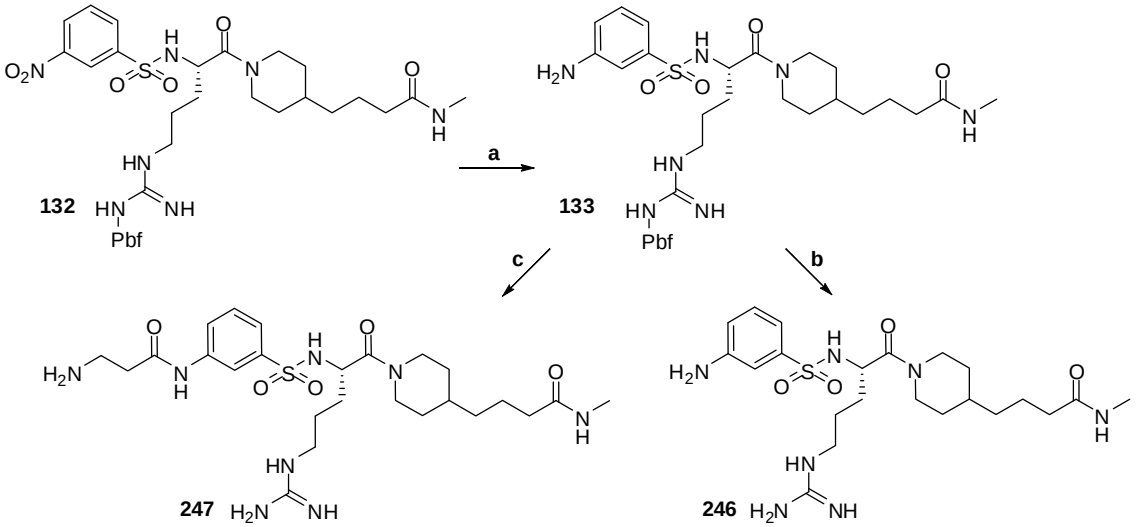


No.	K_i (μM)		
	matriptase	**thrombin**	**fXa**
246 MI-0401	25.4	8.1	>150
247 MI-0402	0.7	25.5	141
248 MI-0407	>150	2.7	22
249 MI-0408	>150	21.5	69

Synthesis of inhibitors with modified P1-residue

The inhibitors 246 and 247 were prepared from compound 132 (Scheme3.5), which was obtained from reaction of 3-nitrophenylsulfonyl chloride with H-Arg(Pbf)-OH under Schotten-Baumann conditions, followed by coupling of *N*-methyl-4-(piperidin-4-yl)butanamide (intermediate 4) using PyBOP.[78] The nitro group was reduced by zinc dust in 90% HOAc providing intermediate 133. This intermediate was coupled with Boc-β-alanine using the mixed anhydride procedure, followed by the removal of the

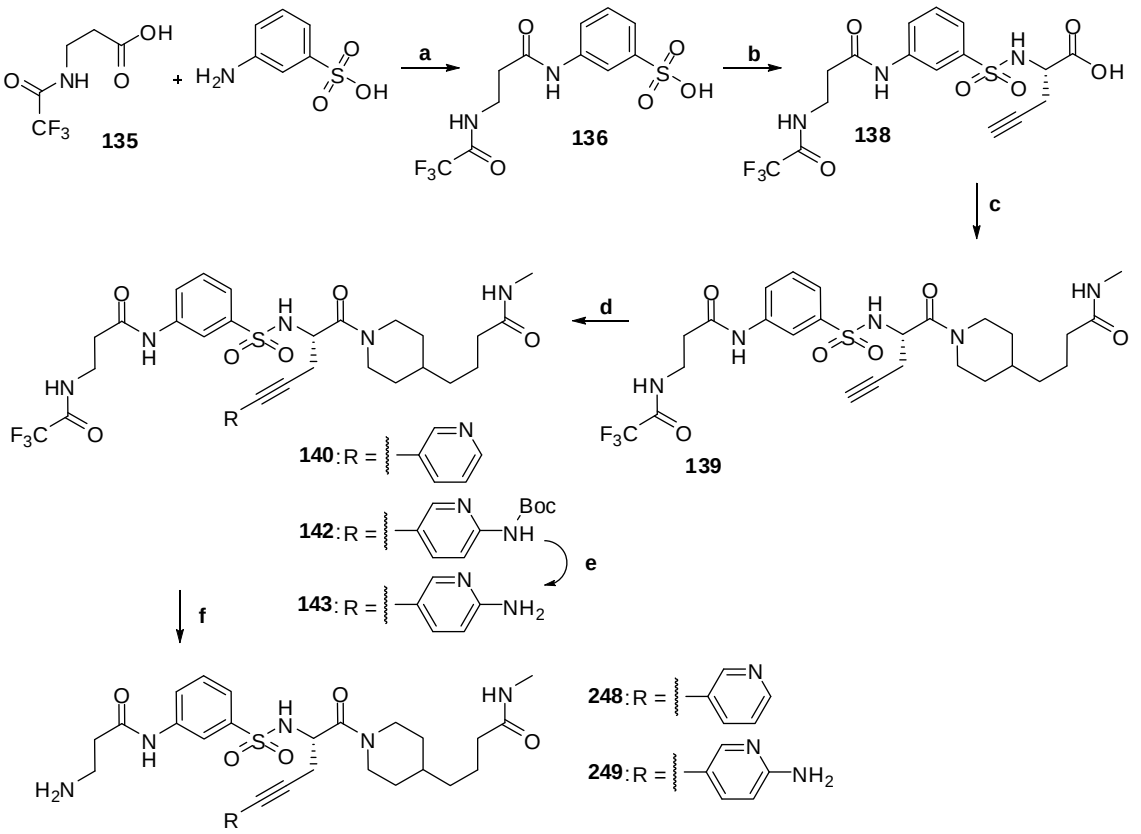
whole protecting group using 90% TFA providing inhibitor 247. Analogue 246 was obtained by direct treatment of intermediate 133 with 90% TFA.



Scheme 3.5 Synthesis of inhibitors **246** and **247.** Reagents and conditions: (a) zinc dust in 90% HOAc 2 h at RT; (b) 90% TFA, at RT 2 h.; (c) i: 1 equiv Cbz-*β*-alanine, 1 equiv isobutyl chloroformate and 1 equiv NMM, 10 min at -15 °C in DMF; 1 equiv intermediate **133**, 1 equiv NMM stirring 1 h at -15 °C and overnight at RT; ii: 90% TFA, at RT 1 h.

The synthesis of the inhibitors **248** and **249** was performed according to Scheme 3.6. The Tfa-*β*-alanine **135** was coupled with metanilic acid (predissolved in DMF in the presence of 1 equiv NMM) using the mixed anhydride procedure. The sulfonic acid was converted into the sulfonyl chloride using 1.2 equiv cyanuric chloride and 1.3 equiv TEA in dry aceton to obtain intermediate **137**.[78] This sulfonyl chloride intermediate was coupled to L-propargylglycine in a mixture of ACN and water in the presence of DIPEA providing compound **138**. This was coupled with *N*-methyl-4-(piperidin-4-yl)butanamide (intermediate **4**) using PyBOP to obtain compound **139**.

A Sonogashira cross coupling was used to prepare intermediates **140** and **142**. For these reactions, 0.025 equiv CuI, 8 equiv TEA, 1 equiv 3-iodopyridine or 2-(Boc-amino)-5-iodopyridine (**141**) and 0.025 equiv tetrakis(triphenylphosphine)palladium (0) were suspended in ACN and refluxed under argon atmosphere for 1 h. This mixture was treated with 1 equiv intermediate **139**, dissolved in ACN, and refluxed under argon atmosphere for an additional 18 h. Compound **142** was deprotected in 50% TFA in DCM. The Tfa-group from compounds **143** and **140** was removed by treatment with 1 N NaOH in a mixture of dioxane/water (pH ≈ 12) and was stirred for 3 h at RT, providing inhibitors **248** and **249**.



Scheme 3.6 Synthesis of inhibitors **248** and **249**. Reagents and conditions: (a) 1 equiv Tfa-*β*-alanine (**135**), 1 equiv isobutyl chloroformate and 1 equiv NMM, 10 min at -15 °C in DMF; 1 equiv metanilic acid, 1 equiv NMM stirring 1 h at -15 °C and overnight at RT; (b) i: 1 equiv intermediate **136** in dry acetone, 1.3 equiv TEA, 1.2 equiv cyanuric chloride, 0.05 equiv 18-crown-6, 20 h reflux; ii: 1 equiv L-propargylglycine, 2 equiv DIPEA, 1 equiv sulfonyl chloride intermediate **137** in a mixture of ACN/water; (c) 1.1 equiv intermediate **4**, 1.05 equiv PyBOP and 3 equiv DIPEA in DMF, 30 min at 0 °C, and 2 h at RT; (d) 1 equiv 3-iodopyridine or 1 equiv 2-(Boc-amino)-5-iodopyridine (**141**), 0.025 CuI, 0.025 equiv tetrakis(triphenylphosphine)palladium (0), 8 equiv TEA in ACN,1 h reflux under argon, 1 eqiuv **139** 18 h reflux in ACN under argon; (e) DCM and TFA (1:1, v/v), stirring 1 h; (f) 1 N NaOH in dioxane/water, pH 12 at RT 3 h, neutralization by 10% TFA.

3.3 D-Phenylglycine derivatives

Derivatives with a central D-phenylglycine residue have been previously described as potent fXa inhibitors (Fig. 3.4). In those initial structures, the amino group of the D-phenylglycine was acylated by a P1 3-amidinobenzoyl residue, whereas its carboxyl group was coupled with substituted piperazines or piperidines, which are supposed to bind into the hydrophobic S3/4 binding site.[202, 203] Due to some structural similarity between matriptase and fXa, this structure type was used for evaluation as matriptase inhibitors.[204] The major differences between both proteases exist in the S3/4 pocket. In fXa this pocket has a box-shaped hydrophobic/aromatic cavity lined by aromatic side chains of Tyr99, Phe174 and Trp215,[205-207] whereas this binding site in matriptase is formed by three aromatic amino acids, Phe97 on the top, Trp215 at the bottom, surrounded by Phe99 on the right and Gln175 on the left side (section 2.2.1).

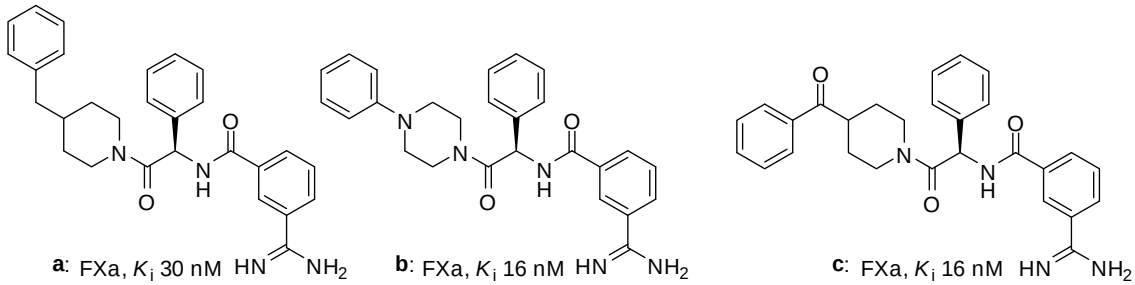
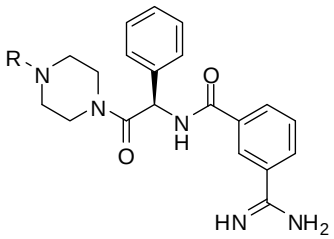
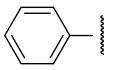
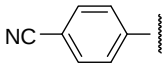
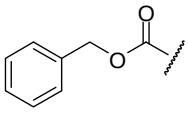
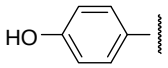
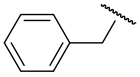
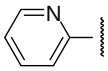
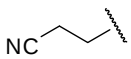
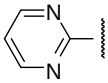
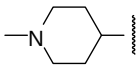
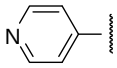
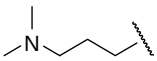
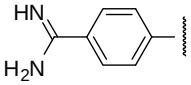
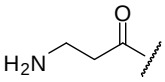
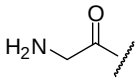
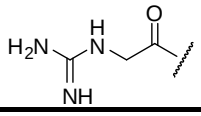


Fig. 3.4 Structures of known fXa inhibitors containing a central D-phenylglycine.[203, 204]

The synthesized inhibitors of this type (Table 3.5) vary only in their substituted piperazine residue that should bind into the S3/4 pocket of matriptase, whereas the 3-amidinobenzoyl-D-phenylglycyl segment was maintained.

Table 3.5: Inhibition of matriptase and related trypsin-like serine proteases by D-phenylglycyl-derived inhibitors with the general formula.



No.	R	K_i (μM)		
		matriptase	thrombin	fXa
250 MI-0415		18	4.1	0.02
251 MI-0418		18	5.3	0.001
252 MI-0420		46	4.6	0.48
253 MI-0421		29	9.2	0.003
254 MI-0426		25	4.5	0.04
255 MI-0422		1.7	4.2	0.023
256 MI-0423		7.4	6.7	0.04
257 MI-0427		2.9	8.1	0.07
258 MI-0416		1.4	29	0.09
259 MI-0417		0.29	17	0.003
260 MI-0424		4.5	3.5	0.024
261 MI-0419		5.6	4.8	0.02
262 MI-0429		12	18	0.50
263 MI-0430		31	21	3.13
264 MI-0435		21	12	3.21

In general, all compounds containing the central D-phenylglycine residue (Table 3.5) are weak to moderate matriptase inhibitors, while some of them show high potency against

fXa, as known from literature.[203, 204] The best matriptase inhibition was found for the pyridin-4-yl inhibitor **259** with a K_i value of 0.29 μM. However, this compound is a much stronger fXa inhibitor, having a K_i value of 3 nM. A 6-10 fold reduced potency was found for the pyridin-2-yl analogue **255**, and the pyrimidin-2-yl derivative **257** with matriptase K_i values of 1.7 and 2.9 μM, respectively. However, both analogues are also strong fXa inhibitors with K_i values $\leq$ 0.07 μM. The 1-methylpiperidin-4-yl compound **258** inhibits matriptase with a K_i value 1.4 μM similarly to analogue **255**.

By contrast, a replacement of the terminal pyridine by phenyl (**250**), benzyl (**254**) or by substituted phenyl (**251** and **253**), results in more than a 50-fold less matriptase inhibition ($K_i \geq 18$ μM). The conversion, in inhibitor **251**, of a nitrile into an amidino residue (**261**) slightly improved the inhibitory potency against matriptase (K_i value 5.6 μM). Inhibitors **262** and **263**, containing the N-terminal β-alanine amide and glycine amide, also show poor potency against matriptase (K_i values 12 and 31 μM, respectively). The conversion of the N-terminal amino group of compound **263** into guanidine provided analogue **264** possesing a slightly increased matriptase affinity (K_i value 21 μM). All of these D-phenylglycine derivatives, summarized in Table 3.5, have poor affinity to thrombin with K_i values > 3.5 μM, whereas some compounds are excellent fXa inhibitors with inhibition constants in the nanomolar range, like compound **251** (K_i value 1 nM for fXa).

It is known that a 2-chlorothiophene carbonyl group is a very suitable P1 residue for fXa inhibitors,[208, 209] which was used for the development of the clinically approved fXa inhibitor Rivaroxaban (Fig. 3.5).[210] This chlorothiophene carbonyl residue was also incorporated in fXa inhibitors containing a central amino acid in D-configuration like D-phenylglycine.[208, 209]

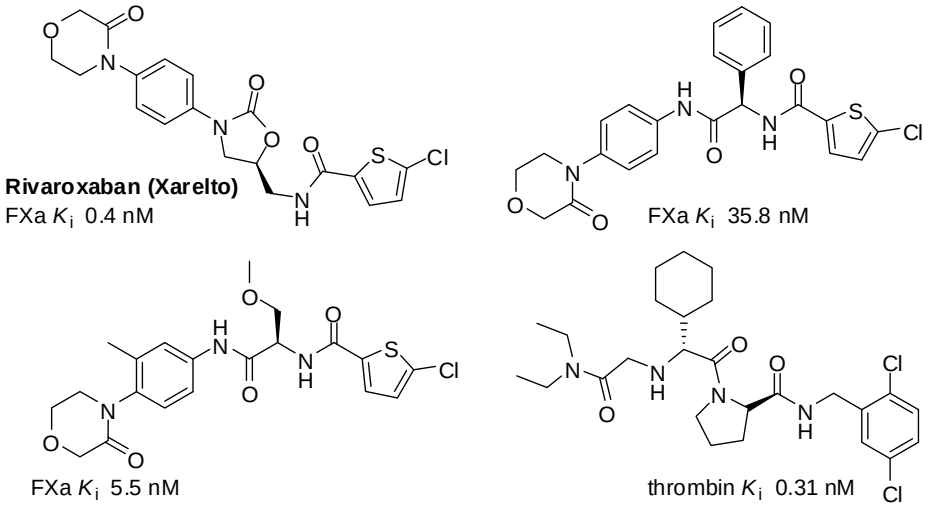
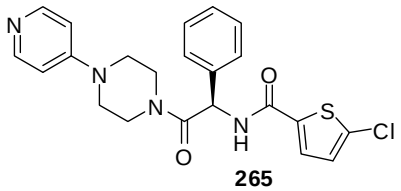


Fig. 3.5 Structures of known fXa and thrombin inhibitors containing 2-chlorothiophene and chloro-aromatic P1 residues.[209-211]

Therefore, the analogous inhibitor **265** (Fig. 3.6) was synthesized, but this compound showed negligible matriptase affinity.



265
MI-0425

	K_i (μM)		
	matriptase	thrombin	FXa
	182	25.6	0.17

Fig. 3.6 Structure of the 2-chlorothiophene-containing inhibitor **256**.

According to present knowledge, these chloro-aromatic groups are only suitable as P1 residues for inhibitors of the related trypsin-like serine proteases thrombin[211, 212] and fXa,[208, 209] which contain an alanine in position 190 next to Asp189 at the bottom of the S1 site. However, not all trypsin-like serine proteases containing an Ala190 can be efficiently addressed by such inhibitors containing a chloro-aromatic P1 residue, like plasma kallikrein (personal information, Prof. Steinmetzer) and HAT.[213] For instance, it was found that substrate analogues containing chloro-substituted benzylamides in P1 position are relatively weak inhibitors of plasma kallikrein and HAT compared to 4-amidinobenzylamide analogues.

3.3.1 Modeled complex of inhibitor 259 in matriptase

In the solved X-ray structure of trypsin in complex with 2-(4-benzoyl-Pip)-D-Phg-3-benzamidine (leb2. pdb-code, inhibitor **c** Fig. 3.4), the benzamidine was found to occupy the S1 pocket making a bidentate salt bridge to Asp189. The phenyl portion of the phenylglycine linker was located above the disulfide bridge between Cys220 and Cys191 and the benzoyl piperidine group binds into the S3/4 site with the carbonyl pointing upward into the solvent.[203, 204] This trypsin/inhibitor complex was superimposed by an rmsd fit to the C_α-atomic coordinates of matriptase (2gv6. pdb-code), followed by replacement of the benzoyl group by a 4-pyridine ring used in inhibitor **259**, which has similar structure to compound **c**. This matriptase/inhibitor 259 complex was energy minimized using Moe program and the fold setting (Fig. 3.7). The modeled structure of this complex shows that the N-terminal 4-pyridine-piperazine group of compound **259** fits well into the S3/4 pocket making a close edge to face contact to the aromatic side chain of Phe99 of matriptase, whereas all characteristic H-bonds of the P1 3-benzamidine were found as known from the complex of inhibitor **c** (Fig. 3.4) in trypsin.

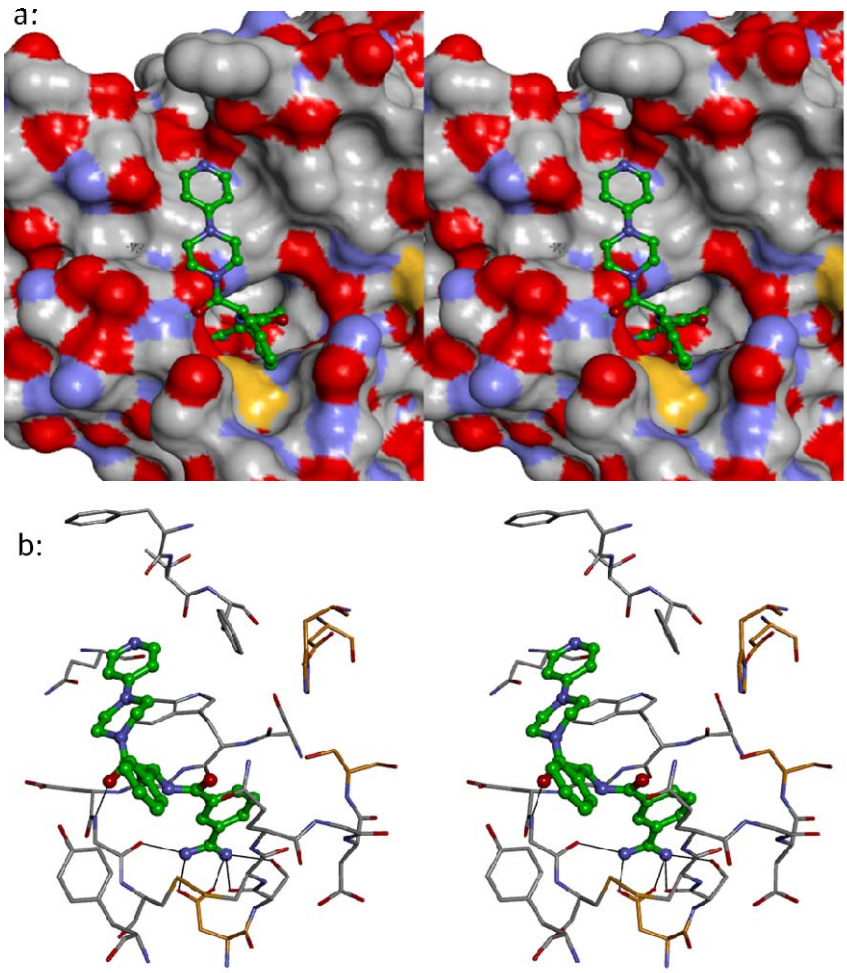


Fig. 3.7 Stereo view of the modeled complex of inhibitor **259** in matriptase. a) Matriptase is shown with its surface colored by atom type (carbon in grey, oxygen in red, nitrogen in blue and sulfur in yellow). The inhibitor is shown in sticks with green carbon atoms. b) Stereo view of the modeled complex showing the hydrogen bonds as black lines, formed between **259** and matriptase residue Asp189, Ser190 and Gly219.

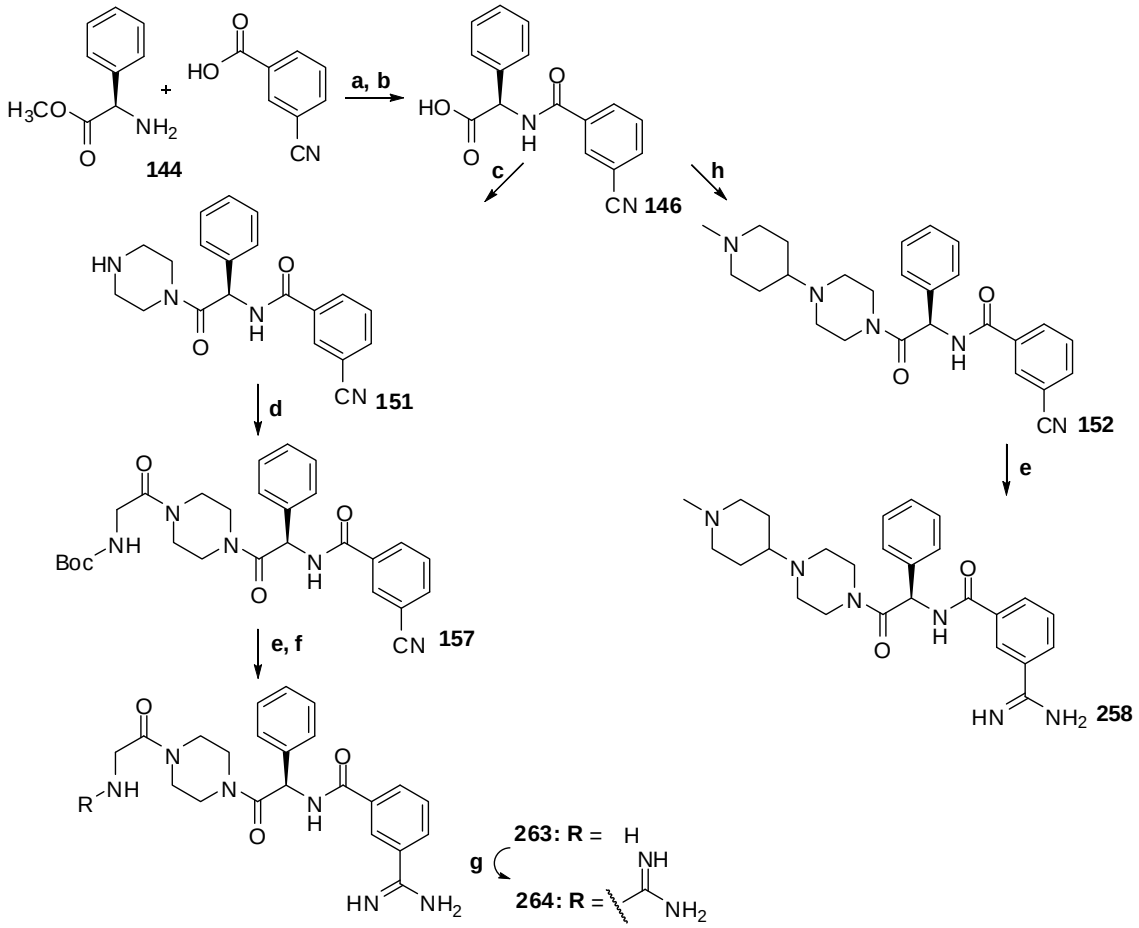
3.3.2 Synthesis of D-phenylglycine derivatives

The synthesis of the inhibitors shown in Table 3.5 was performed according to previously described methods using intermediate **146**.[204]

D-Phenylglycine was treated with thionyl chloride in MeOH to perform its methyl ester, which was coupled with 3-cyanobenzoic acid using the mixed anhydride procedure, followed by saponification to obtain intermediate **146**. This compound was first coupled with Boc-piperazine, followed by deprotection using 1 N HCl in HOAc and coupling of Boc-*β*-alanine. The nitrile-containing compound was converted into the amidine according to the procedure of Judkins,[188] as described in Scheme 3.7. The final inhibitor **263** was obtained after Boc-deprotection, which was further converted into the

guanidine analogue **264** by treatment with 1-*H*-pyrazole-carboxamidine and DIPEA in DMF.[214]

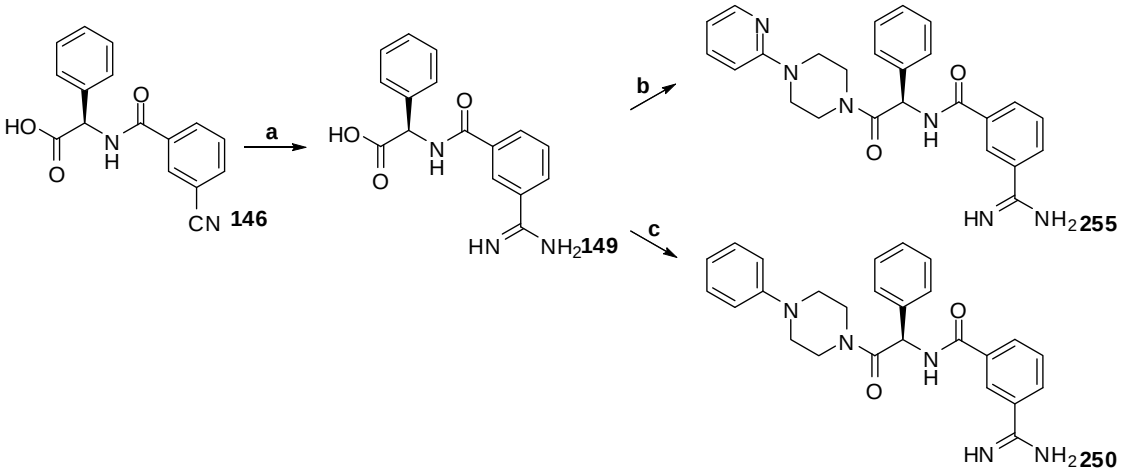
Intermediate **152** was prepared by the mixed anhydride coupling of compound **146** with 1-(1-methylpiperidin-4-yl)piperazine, followed by conversion of the cyano group into amidine as described previously, to obtain inhibitor **258** (Scheme 3.7).



Scheme 3.7 Synthesis of inhibitors **258, 263** and **264**. Reagents and conditions: (a) 1 equiv 3-cyanobenzoic acid, 1 equiv isobutyl chloroformate and 1 equiv NMM, 10 min at -15 °C in DMF; 1 equiv intermediate **144**, stirring 1 h at -15 °C and overnight at RT; (b) 1 N NaOH in dioxane and water, pH 12, 3 h at RT, acidified by 5% $KHSO_4$-solution; (c) i: 1.1 Boc-piperazine, mixed anhydride procedure, see step a; ii: 1 N HCl in glacial HOAc, 1 h at RT; (d) 1 equiv Boc-*β*-alanine, 1.25 equiv EDCI, 1.1 equiv HOBT in DMF, 3 h at RT (e) i: 3 equiv hydroxylamine x HCl and 3 equiv DIPEA, 4 h reflux in ethanol, and stirring overnight at RT; ii: 3 equiv Ac_2O in HOAc, 30 min at RT; iii: H_2 and Pd/C as catalyst in 90% HOAc, stirring overnight at RT; (f) 90% TFA, at RT 1 h; (g) 2 equiv 1-*H*-pyrazole-carboxamidine x HCl, 2 equiv DIPEA in DMF stirring 24 h at RT; (h) 1.1 equiv 1-(1-methylpiperidin-4-yl)piperazine, mixed anhydride procedure, see step a.

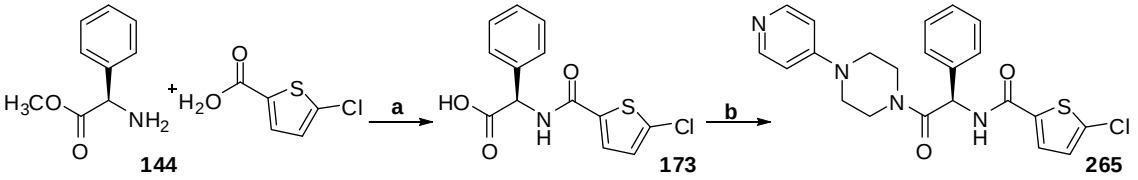
Initially, the nitrile **146** was directly converted into the amidine **149** and coupled with 1-(2-Pyr)piperazine to obtain inhibitors **250** and **255** (Scheme 3.8).

We assumed that this strategy is more convenient for the preparation of a large inhibitor series of this type, since this would require only a single amidine preparation. However, this procedure provided only poor yields with several side products during the coupling of the free amidino intermediate **149** with the used piperazine derivatives. Therefore, most of the inhibitors of this series were prepared as described in Scheme 3.7.



Scheme 3.8 Synthesis of inhibitors **250** and **255**. Reagents and conditions: (a) i: 3 equiv hydroxylamine x HCl and 3 equiv DIPEA, 4 h reflux in ethanol, and stirring overnight at RT; ii: 3 equiv Ac_2O in HOAc, 30 min at RT; iii: H_2 and Pd/C as catalyst in 90% HOAc, stirring overnight at RT; (b) 1 equiv 1-(2-Pyr)piperazine, 1.25 equiv EDCI, 1.1 equiv HOBT in DMF, stirring under argon atmosphere, 3 h at RT; (c) 1 equiv 1-phenylpiperazine, 1.05 equiv PyBOP and 3 equiv DIPEA in DMF, 30 min at 0 °C, and 2 h at RT.

Inhibitor **265** was synthesized by reaction of compound **144** with 5-chlorothiophene-2-carboxylic acid using the mixed anhydride procedure, followed by saponification and coupling of 1-(pyridin-4-yl)piperazine (Scheme 3.9).



Scheme 3.9 Synthesis of inhibitor **265**. Reagents and conditions: (a) i: 1 equiv 5-chlorothiophene-2-carboxylic acid, 1 equiv isobutyl chloroformate and 1 equiv NMM, 10 min at -15 °C in DMF; 1 equiv intermediate **144**, stirring 1 h at -15 °C, and overnight at RT; ii:1 N NaOH in dioxane and water, pH 12, 3 h at RT, acidification by 5% $KHSO_4$-solution; (b) 1-(pyridin-4-yl)piperazine, mixed anhydride procedure, see step a.

3.4 Antranilamide derivatives

From an X-ray crystallographic screen, the clinically used antiarrythmic drug Mexiletine was identified as a weak uPA inhibitor. It was found that its terminal amino group binds into S1 pocket of uPA, makes a salt bridge to Asp189 and hydrogen bonds to the carbonyls of Gly219 and Ser190. Further modifications resulted in a series of substituted anilides of 4-(2-aminoethoxy)benzoic acid with strongly improved uPA affinity (Fig. 3.8).[215] An additional known uPA inhibitor is amiloride containing an acylated guanidine that also occupies the S1 binding site.

These compounds served as a template for the synthesis of para-(guanidinomethyl)-benzoyl antranilamide derivatives.

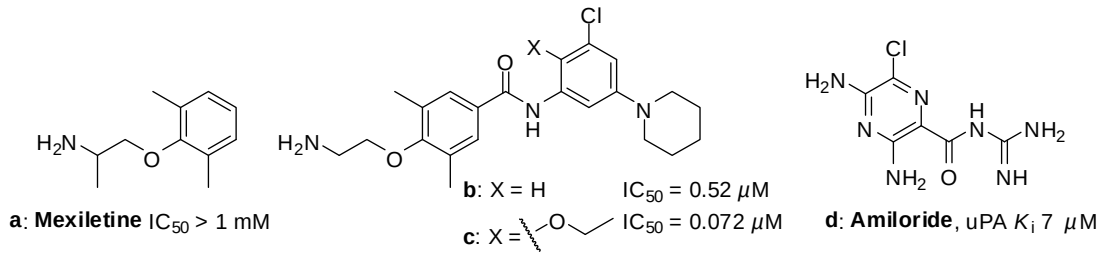
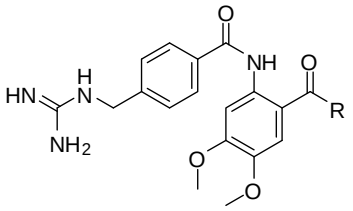


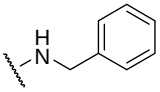
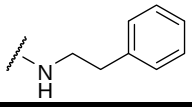
Fig. 3.8 Structures of non peptidic uPA inhibitors.[215, 216]

Due to significant structural similarity between matriptase and uPA in their S1 pocket, which are both Ser190 proteases, this structure type was used for evaluation of matriptase inhibitors. By contrast, the major differences between both proteases exist in the S3/4 pocket. Urokinase possesses a two amino acid insertion of Thr97A and Leu97B,[217] which partially reduces the size of the S3/4 pocket, whereas matriptase has a larger S3/4 pocket than found in uPA. Therefore, the ethoxy group in the known uPA-inhibitors (compound **c**, Fig. 3.8)[215] was replaced by larger groups, which should interact with this binding pocket. Furthurmore, the aminoethoxy group was replaced by a guanidinomethyl residue analogue to the acylguanidine of amiloride (**d**, Fig.3.8), which has a similar length and enable a binding to Asp 189 in the S1 pocket.

The synthesized inhibitors of this type (Table 3.6) vary in the length of the residue that should bind into the S3/4 pocket of matriptase.

Table 3.6: Inhibition of matriptase and related trypsin-like serine proteases by inhibitors of the general formula.

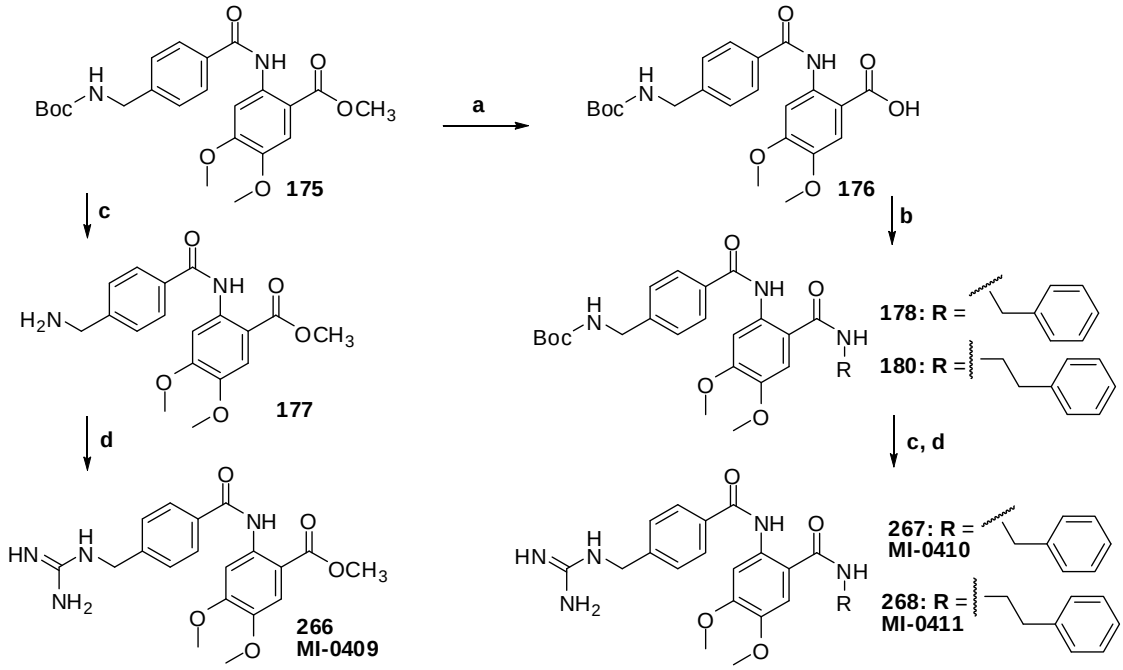


No.	R	K_i (μM)			
		matriptase	thrombin	fXa	uPA
266 MI-0409	OCH_3	73	26	103	> 30
267 MI-0410		35	27	2.2	> 100
268 MI-0411		31	7.3	77	> 100

However, inhibitors **266**-**268** possess a weak potency against matriptase with K_i values > 30 μM. These three compounds are also weak thrombin, fXa and uPA inhibitors. Therefore, no further derivatives in this series were prepared.

Synthesis of anthranilamide derivatives

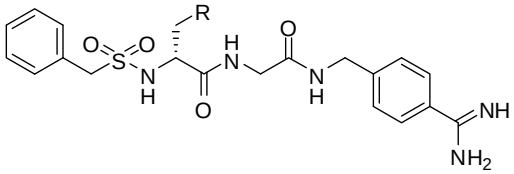
4-(Boc-aminomethyl)benzoic acid was coupled with commercially available methyl 2-amino-4,5-dimethoxybenzoate by the mixed anhydride procedure to obtain intermediate **175**. Deprotection using 1 N HCl in HOAc provided compound **177**, which was further converted into guanidine **266** by treatment with 1-*H*-pyrazole-carboxamidine and DIPEA in DMF.[214] Alternatively, intermediate **175** was saponified and coupled with benzylamine or phenylethylamine using the mixed anhydride procedure to obtain compounds **178** and **180**. After removal of the Boc-group, amine intermediates were converted into guanidino analogues **267** and **268** by treatment with 1-*H*-pyrazole-carboxamidine.[214]



Scheme 3.10 Synthesis of inhibitors **266-268**. Reagents and conditions: (a) 1 N NaOH in dioxane and water, pH 12, 3 h at RT, acidified by 5% $KHSO_4$-solution (b) 1 equiv isobutyl chloroformate and 1 equiv NMM, 10 min at -15 °C in DMF; 1 equiv benzylamine or 1 equiv phenylethylamine, stirring 1 h at -15 °C, and overnight at RT; (c) 1 N HCl in glacial HOAc, 1 h at RT; (d) 2 equiv 1-*H*-pyrazole-carboxamidine x HCl, 2 equiv DIPEA in DMF stirring 24 h at RT.

3.5 Substrate analogue inhibitors

Substrate analogues containing a C-terminal 4-amidinobenzylamide as a decarboxylated P1 arginine mimetic are potent inhibitors of various trypsin-like serine proteases, like thrombin, factor Xa, factor VIIa, plasma kallikrein, and uPA.[90, 162-164, 218-220] Such inhibitors are stable against degradation by carboxypeptidases and in case of the N-terminal acylation or sulfonylation they are also resistant against aminopeptidases. Most of these inhibitors possess a P3 amino acid in D-configuration. There exist only a few potent examples as thrombin inhibitors containing a P3 residue in L-configuration.[221, 222] Selectivity studies with previously described substrate analogue inhibitors of fXa revealed that some of them containing D-homophenylalanine analogues in P3 position (**9**-**10**) also possess a moderate affinity to matriptase with K_i values > 50 nM (Fig. 3.9).[161]



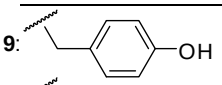
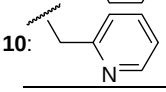
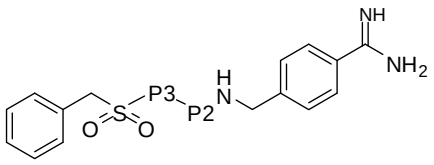
	R	K_i (nM) matriptase	thrombin	FXa	trypsin
9:		70	1500	3.0	3.9
10:		110	1900	0.71	7.7

Fig. 3.9 Known substrate analogue inhibitors of trypsin-like serine proteases.[161]

The very non-specific analogue MI-0001, containing a D-arginine as P3 residue has been previously described as a potent fXa, thrombin and plasma kallikrein inhibitor,[223] whereas the D-cyclohexyl alanine analogue MI-0003 was characterized as a highly potent thrombin inhibitor.[183, 224] Both analogues were also described as first low molecular weight matriptase-2 inhibitors with K_i values of 0.17 μM and 0.46 μM, respectively. However, they are also slightly stronger matriptase inhibitors.[178]

Therefore, an extended screen of matriptase with additional substrate analogues, which were available in our group from previous studies[178, 183] was performed. All of these inhibitors contain a C-terminal 4-amidinobenzylamide and an N-terminal benzylsulfonyl group, but vary in their P2 and P3 residues (Table 3.7).

Table 3.7: Screening of substrate-analogue inhibitors of related trypsin-like serine proteases with matriptase, which were previously described.[178, 183, 223]



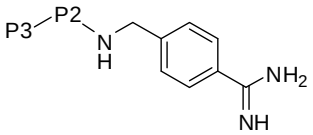
No.	P3	P2	K_i (μM)			
			matriptase	Thrombin	fXa	HAT
MI-0320	D-hPhe	Pro	0.013	0.001	0.014	0.063
MI-0323	D-hArg	Pro	0.03	0.0004	0.003	0.013
MI-0001	D-Arg	Pro	0.033[a]	0.004	0.0029	0.019
MI-0322	D-Lys	Pro	0.04	0.001	0.052	0.04
MI-0326	D/l-hAla(2Pyr)	Pro	0.073	0.001	0.0022	0.041
MI-0321	D-Lys(Cbz)	Pro	0.2	0.0004	0.017	0.053
MI-0361	D-Phe(4-Am)	Pro	0.3	0.003	0.15	0.17
MI-0003	D-Cha	Pro	0.33[a]	0.0001	0.035	0.11
MI-0317	D-Phe-	Pro	0.34	0.0001	0.077	0.098
MI-0354	D-Val	Pro	0.77	0.0013	0.012	0.076
MI-0329	D-Ala	Pro	2.76	0.005	0.83	n.d
MI-0328	Gly	Pro	5.91	0.004	5.17	n.d
MI-0371	D-Arg	Ala	0.02	0.075	0.001	0.03
MI-0360	D-Arg	Arg	0.023	n.d.	n.d.	0.46
MI-0381	D-Arg	Abu	0.065	0.029	0.002	0.014
MI-0380	D-Arg	Nva	0.12	0.068	0.037	0.015
MI-0366	D-Arg	Ser	0.2	0.5	0.02	0.034
MI-0377	D-Arg	Leu	0.46	n.d.	n.d.	0.05
MI-0378	D-Arg	Val	0.57	0.66	0.021	0.022
MI-0379	D-Arg	Phe	0.24	n.d.	n.d.	0.06
MI-0324	Lys	Lys	2	n.d	n.d	0.2
MI-0365	D-Phe	Ser	4.3	0.02	0.24	0.18
MI-0362	D-Phe	Lys(Cbz)	15	0.0006	0.7	0.67

[a]These matriptase inhibition constants have been previously described.[178]

In a first series, a proline was maintained as P2 residue, whereby various D-amino acids were incorporated in P3 position. All of these analogues are highly potent thrombin inhibitors with K_i values ≤ 5 nM, because proline is a preferred P2 residue for this trypsine-like protease.[225] **MI-0320** was the most potent matriptase inhibitor, which could be identified by this screening, possessing a K_i value of 13 nM. This compound contains a D-homophenylalanine as P3 residue, similar to several homoamino acids in the D-configuration were used for compounds **9-10** (Fig. 3.9). A slightly reduced affinity with K_i > 100 nM was found for analogues containing D-homoarginine, D-lysine, D/L-homo-2-pyridylalanine and D-arginine as previously reported.[178] The found

inhibition constants confirm previous results obtained with the 3-amidinophenylalanine-derived inhibitors that matriptase preferably accommodates aromatic or basic groups in its S3/4 pocket. A second series of those previously synthesized analogues contains a constant P3 D-arginine, whereas the P2 residue was modified. Among these inhibitors, compounds **MI-0371** and **MI-0360** containing P2 alanine and arginine, respectively, have the strongest potency against matriptase; with K_i value close to 20 nM. The replacement of P2 proline with other amino acids generally reduces the affinity to thrombin. However the alanine derivative **MI-0371** and aminobutyric acid derivative **MI-0381** were found to be excellent fXa inhibitors ($K_i \leq 2$ nM).[90, 218, 226] All other analogues showed a relatively weak matriptase inhibition. Previous studies with related substrate analogue inhibitors revealed that the presence of an N-terminal benzylsulfonyl group such as in Bzls-D-Ser(tBu)-Gly-4-amidinobenzyl-amide (a K_i value for fXa 14 nM) considerably increases the affinity to fXa by a 1000-fold, compared to an analogue missing the P4 residue (H-D-Ser(tBu)-Gly-4-amidinobenzyl-amide, K_i value for fXa 16 μM). However, this effect was less pronounced with other trypsine-like serine proteases, like thrombin, plasmin, uPA, and trypsin,[218] although the Bzls-derivatives were also more potent with these proteases. To reduce the affinity to fXa, several new substrate analogue inhibitors without Bzls-group were prepared (Table 3.8).

Table 3.8: Inhibition of matriptase and related trypsin-like serine proteases by substrate analogue inhibitors.



No.	P3	P2	K_i (μM)		
			matriptase	thrombin	fXa
273 MI-0464	H-hPhe	Pro	19.65	7.61	66.36
274 MI-0465	H-Phe	Pro	46.5	17.64	212.31
275 MI-0466	H-D-Phe[a]	Pro	3.89	0.004	37.53
276 MI-0467	H-D-hPhe	Pro	0.06	0.072	2.58
279 MI-0468	H-D-Arg	Pro	0.25	0.69	0.74
277 MI-0469	H-D-hPhe	Ala	0.044	0.73	1.79
278 MI-0470	H-D-hTyr	Ala	0.026	0.30	0.57

[a]The inhibitor was previously described as thrombin inhibitor possessing a K_{ass} of 6.8 x 10^8 L/mol.[162]

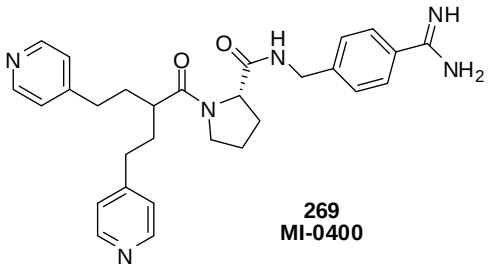
In addition to inhibitors with D-amino acid in P3 position, two analogues with L-configuration were prepared for comparison. However, inhibitors **273** and **274** are completely inactive against matriptase and the related trypsin-like serine proteases.

All compounds are relatively weak fXa inhibitors confirming previous results.[218] The P2 proline derivatives in combination with P3 D-phenylalanine (**275**) and D-homophenylalanine (**276**) retain significant potency against thrombin (K_i values 4 nM and 72 nM, respectively), whereas the D-arginine compound **279** is the first substrate analogue with stronger affinity to matriptase compared to thrombin and fXa.

For further reduced affinity to thrombin, the alanine derivatives **277** and **278** were synthesized. Based on the screening results (Table 3.7), this modification has slightly improved the matriptase inhibition and selectivity. Inhibitor **278** containing D-homotyrosin as P3 residue possesses an inhibition constant of 26 nM against matriptase, which is more than 10-fold stronger compared to the K_i values against thrombin and fXa. Its analogue **277**, containing the P3 -D-homophenylalanine, has also an acceptable affinity to matriptase (K_i 44 nM) compared to thrombin and fXa.

Despite it is known that trypsin-like serine proteases prefer P3 residues in D-configuration,[227] untill now there exist only some examples of thrombin inhibitors with L-amino acids in the P3 position.[221, 222] Therefore, we assumed that it could be useful to incorporate also Cα-dialkylated amino acids in P3 position for further improvement in affinity to matriptase. Such residues can be considered as a kind of chimera between amino acids in D- and L-configuration. However, all of those reported analogues contain two different alkyl groups at the P3 Cα-atom resulting in a mixture of configuration isomers, which requires a laborious purification to obtain the single isomers. To use this strategy and to overcome the synthetic problems, a few analogues containing two identical alkyl groups at the P3 Cα-atom were prepared.

The first compound of this series is inhibitor **269**, which contains 4-(pyridin-4-yl)-2-(2-(pyridin-4-yl)ethyl)butanoyl as an N-terminal acyl residue, and it is missing an amino group. This acyl residue was obtained as a side product during malonic ester synthesis with 4-vinylpyridine to obtain 4-(pyridin-4-yl)butanoic acid,[228] prepared as a precursor for 4-(piperidin-4-yl)butanoic acid, which was incorporated in inhibitors of the 3-amidinophenylalanine type. However, this compound is a relatively weak inhibitor of matriptase, thrombin and fXa (Fig. 3.10).



269
MI-0400

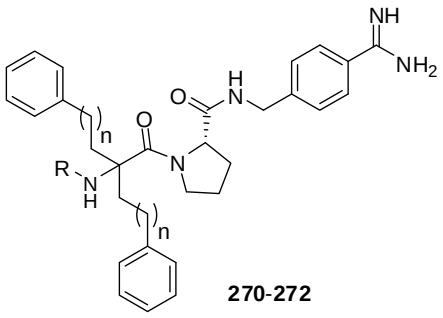
	K_i (μM)		
matriptase	thrombin	fXa
2.7	0.12	4.6

Fig. 3.10 Structure of inhibitor **269**.

We assumed that the weak affinity is mainly related to the missing amino group in the P3 position, which normally interacts with the carbonyl oxygene of Gly216. However, it should be noted that inhibitor **269** contains two 4-pyridinyl groups; therefore, it is difficult to compare this inhibitor with the analogues shown in Tables 3.8 and 3.9.

The structure of the prepared inhibitors containing Cα-dialkylated amino acids are shown in Table 3.9. All of these analogues have a very similar affinity to matriptase as found in inhibitor **269**, whereas their potency against thrombin was significantly enhanced.

Table 3.9: Inhibition of matriptase and related trypsin-like serine proteases by substrate analogue inhibitors of the general formula.



270-272

No.	n	R	K_i (μM)		
			matriptase	thrombin	fXa
270 MI-0457	1	Tfa	6.6	0.086	22
271 MI-0456	0	Tfa	13	0.003	21
272 MI-0459	1	H	0.94	0.042	4.2

The kinetic results for these few examples revealed that the incorporation of Cα-dialkylated P3 amino acids is not a suitable strategy for the design of substrate analogue matriptase inhibitors.

3.5.1 Crystal structure of thrombin in complex with inhibitor 271

Inhibitor **271** (Tfa-Dbzg-Pro-4-Amba) has a strong potency against thrombin with a K_i value of 3 nM. To obtain an impression of its binding mode, it was soaked into thrombin/benzamidine crystals and the structure was solved by X-ray crystallography. As far as we know, this is the first crystal structure of thrombin in complex with a substrate analogue inhibitor containing a Cα-dialkylated P3 amino acid.

The crystal structure shows that one benzyl group is bound into the distal S3/4 aryl binding site, whereas the other benzyl group is directed into the solvent. At present, we can not explain the reason for the more than 4000-fold reduced inhibition of matriptase by inhibitor **271**. However, it should be noted that this preliminary crystal strucrure of the complex thrombin/**271** is not finally resolved till now and therefore, not submitted to the protein data bank.

However, at the present stage of the refinement, the characteristic interactions of the amidine group in the S1 pocket to Asp189, Gly219 and to the characteristic water molecule are found, as well as the hydrogen bond between the carbonyl of Ser214 and the P1 amide NH (Fig. 3.11).

a)

b)

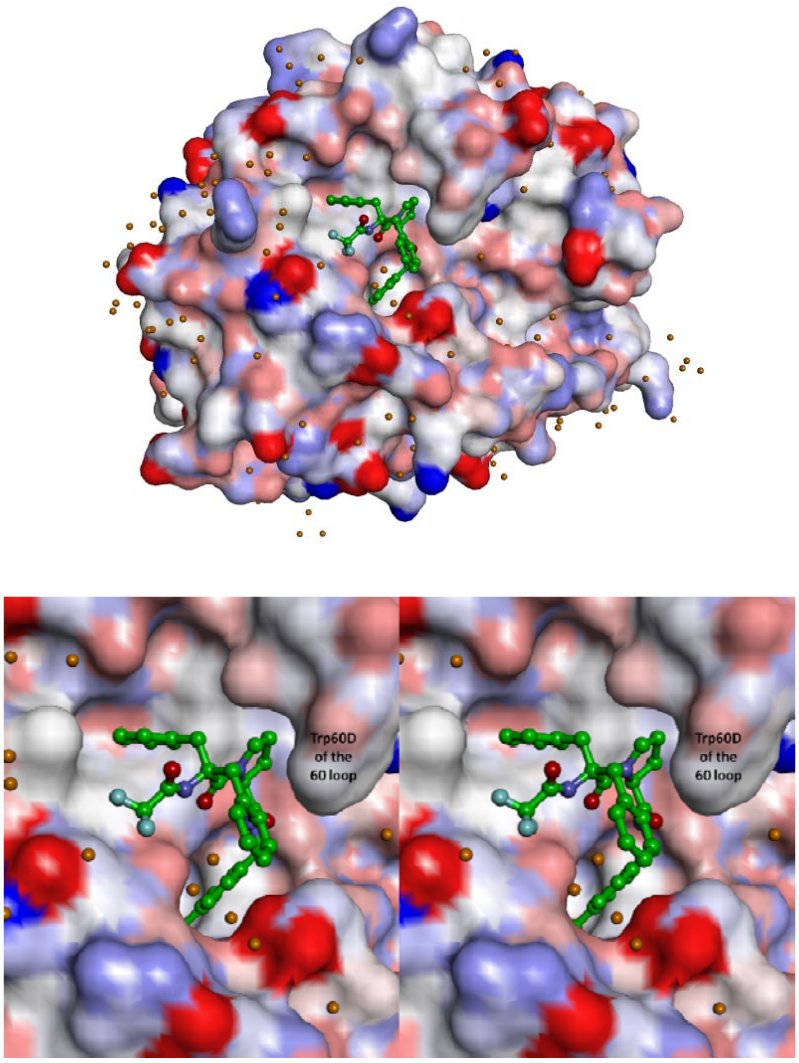
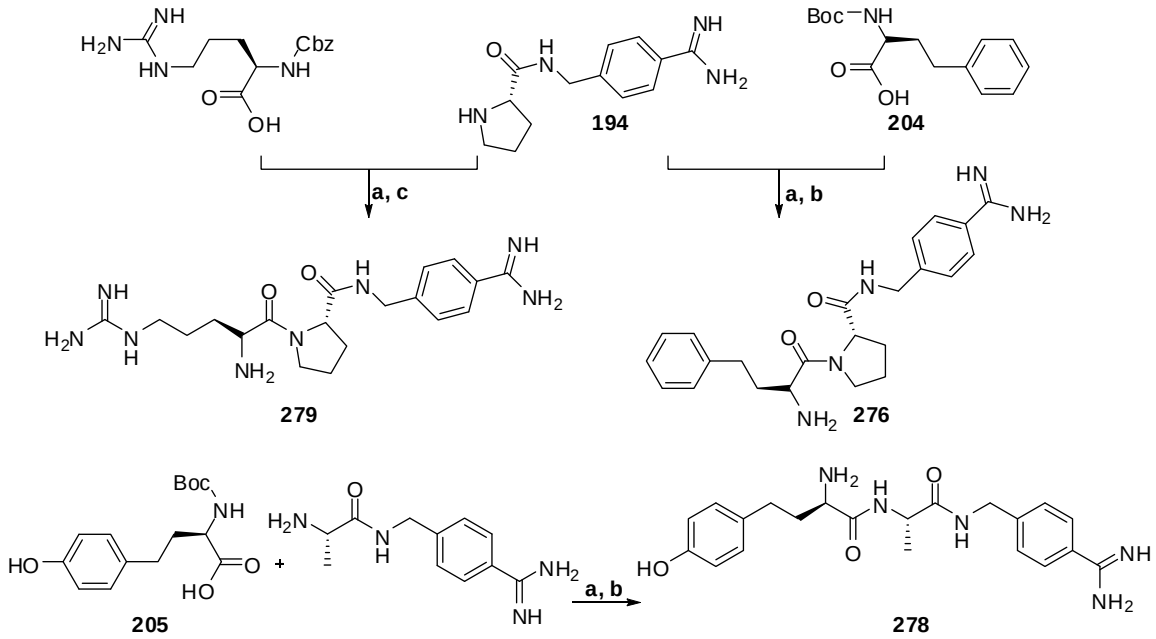


Fig. 3.11 Crystal structure of thrombin in complex with inhibitor **271**. a) Thrombin is shown with its solvent-accessible surface colored by atom charge (negatively charged atoms in red and positively chargeds atom in blue) and inhibitor **271** in sticks. b) Stereo view of the complex in the active site of thrombin.

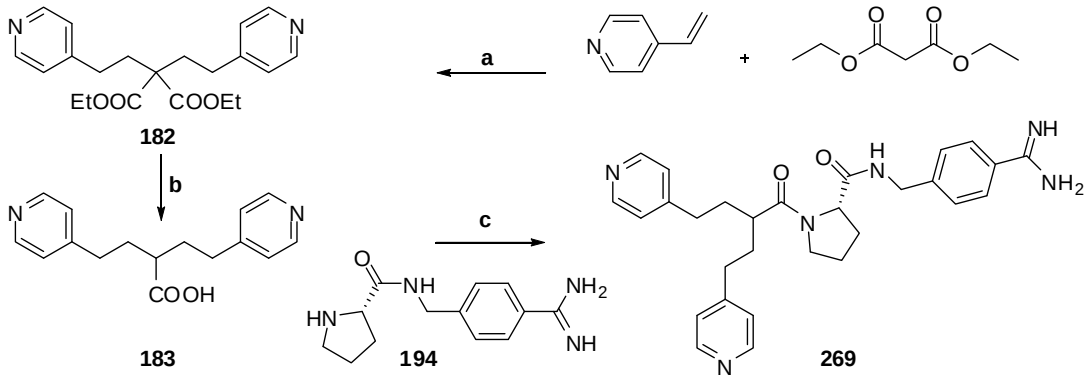
3.5.2 Synthesis of substrate-analogue inhibitors

The synthesis of the inhibitors shown in Table 3.8 was performed according to previously described methods.[90, 178, 218, 221] Cbz-Pro-(4-acetylhydroxyamidino)benzyl-amide[221] (provided by Prof. Steinmetzer) was hydrogenated providing intermediate **194**, whereby the analogue Ala-4-amidinobenzylamide was provided by Dr. Frank Sielaff. These P2-P1 segments were coupled with various *N*-protected amino acids using PyBOP/DIPEA in DMF. Cleavage of the N-terminal protection group provided the final inhibitors **276**, **278** and **279** (Scheme 3.11).



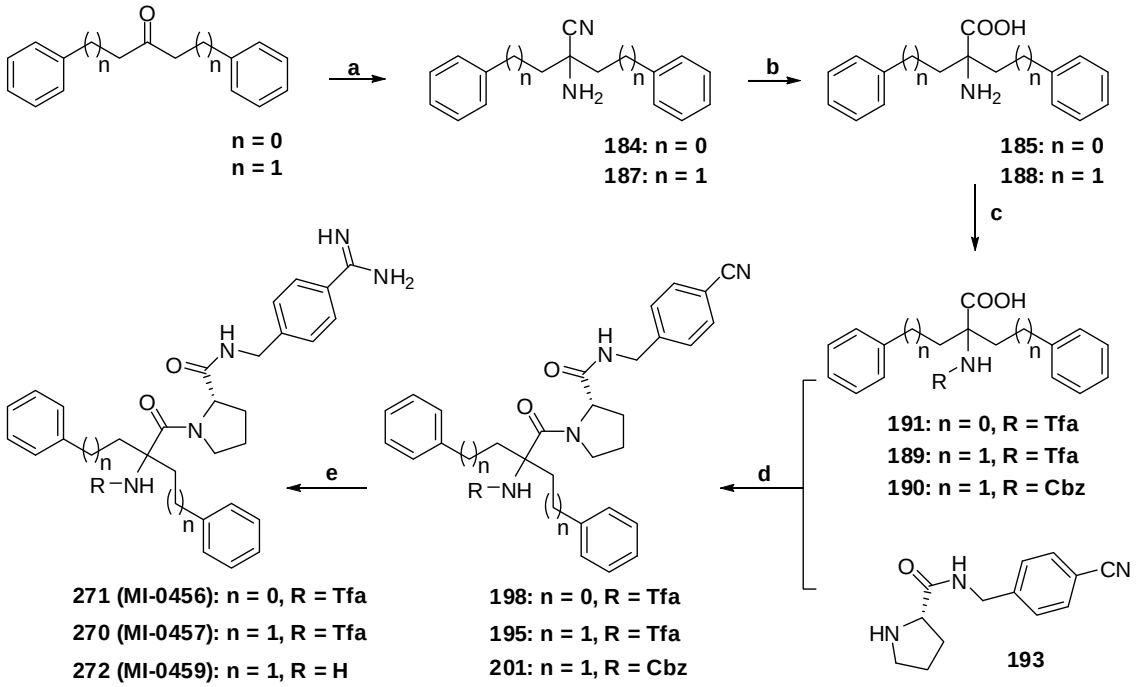
Scheme 3.11 Synthesis of inhibitors **276, 278** and **279**. Reagents and conditions: (a) 1.05 equiv PyBOP and 3 equiv DIPEA in DMF, 30 min at 0 °C, and 2 h at RT; (b) 90% TFA, at RT 2 h.; (c) 32% HBr in HOAc, 1 h at RT.

Inhibitor **269** was synthesized according to Scheme 3.12. Intermediate **182** (diethyl 2,2-bis(2-(pyridin-4-yl)ethyl)malonate) was prepared by treating diethyl malonate with sodium hydride in ethanol, followed by treatment with 4-vinylpyridine according to a previously described procedure for the preparation of 4-(pyridin-4-yl)butanoic acid.[228] To obtain the dialkylated derivative **182**, a high excess of the starting material 4-vinylpyridine was used. This compound was saponified and decarboxylated[229] to obtain intermediate **183**, which was coupled with H-Pro-4-Amba (**194**) producing inhibitor **269**.



Scheme 3.12 Synthesis of inhibitor **269**. Reagents and conditions: (a) 1.7 equiv NaH, in ethanol at 0 °C, 1 equiv diethyl malonate, stirring 30 min at 80 °C, the mixture was cooled, 1.1 equiv 4-vinylpyridine, 3 h reflux; (b) 3 N NaOH in dioxane and water, stirring 7 h at 95 °C, neutralization with 1 N HCl; (c) 1.05 equiv PyBOP and 3 equiv DIPEA in DMF, 30 min at 0 °C, and 2 h at RT.

The Cα-dialkylated P3 amino acids for inhibitors **270**-**272** were synthesized by a modified strecker synthesis using appropriate symmetric ketones as a starting material, according to previously decribed methods.[230] Commercially available 1,3-diphenylpropan-2-one or 1,5-diphenylpentan-3-one **186**, dissolved in MeOH was treated with a solution of NH_4Cl in water, NaCN and ammonia (Scheme 3.13). The mixture was stirred at 50 °C for 32 h, followed by hydrolysing the nitriles **184** and **187** were hydrolized by 6 N HCl to obtain the amino acids di(phenylethyl)glycine **188** or di(benzyl)glycine **185**.[231, 232] These glycine derivatives were protected by treatment with trifluroacetic anhydride in TFA. Alternatively intermediate **188** could also Cbz-protected. The protected dialkyled-glycine derivatives were coupled with H-Pro-4-cyanobenzylamide × HCl (**193**) using HATU/DIPEA in DCM to obtain intermediates **195**, **198** and **201**. These nitriles were converted into amidines as described previously, whereby the Cbz-group was cleaved from intermediate **203** during hydrogenation, to obtain inhibitosr **270**-**272**.



Scheme 3.13 Synthesis of inhibitors **270**-**272**. Reagents and conditions: (a) 3.2 equiv NH_4Cl, 2.9 equiv NaCN, NH_3 in water, 1 equiv 1,3-diphenylpropan-2-one or 1 equiv 1,5-diphenylpentan-3-one dissolved in MeOH, 32 h at 50 °C, acidification with 1 N HCl; (b) ≈ 6 N HCl in water, stirring under reflux for 48 h; (c) i: for Tfa-protection: 2 equiv of trifluoroacetic anhydride in TFA, stirring at 1 h 0 °C, at RT overnight; or ii: for Cbz-protection: 2 equiv NMM, 1.1 equiv Cbz-OSu dissolved in ACN, stirring 1h at 0 °C, 3 h at RT; (d) 1.1 equiv intermediate **193**, 1.25 equiv HATU, 2 equiv DIPEA in DCM, stirring 1 h 0 °C, strirring 2 h at RT; (e) i: 3 equiv hydroxylamine x HCl and 3 equiv DIPEA, 4 h reflux in ethanol, and stirring overnight at RT; ii: 3 equiv Ac_2O in HOAc, 30 min at RT; (f) H_2 and Pd/C as catalyst in 90% HOAc, stirring overnight at RT.

Despite many attempts, it was impossible to remove the Tfa-protection group by treatment with various bases, a similar problem was reported previously.[233]

The N-terminal Tfa-group was used as an amino protecting group, because it was impossible to couple H-Pro-4-cyanobenzylamide × HCl (**193**) to Boc- or Cbz-di(benzyl)glycine (**185**) by various coupling procedures, including the PyBOP, HATU, HBTU, PyBrOP, EDCI, or mixed anhydride method. Several examples have demonstrated that in peptide chemistry, it is difficult to couple amino components to highly hindered dialkylated amino acids, like D-amino isobutyric acid (AIB).[234, 235] By contrast, it was possible to couple the H-Pro-4-cyanobenzylamide × HCl (**193**) to the Cbz-protected di(phenylethyl)glycine (**190**). It seems that this amino acid is sterically less hindered than the analogous di(benzyl)glycine-derivative. However, for the preparation of all these analogues, relatively poor coupling yields were observed.

4 Conclusion and outlook

In the present work, various types of new matriptase inhibitors were developed. These compounds were tested against several trypsin-like serine proteases including matriptase, thrombin and fXa. In some cases additional proteases like uPA, matriptase-2, and HAT were used. Efficient matriptase inhibitors could be useful drugs for the treatment of several deseases like cancer, osteoarthritis, atherosclerosis and few types of influenza.

4.1 3-Amidinophenylalanine derivatives

In a first series, new 3-amidinophenylalanine-derived matriptase inhibitors were synthesized. The strongest derivatives of this type possess an N-terminal 2′,4′-dimethoxy or 2′,4′-dichlorobiphenyl-3-sulfonyl with a C-terminal 2-aminoethylpiperidide residue (section 3.1, Table 3.1). Both dibasic compounds inhibit matriptase with K_i values ≤ 2 nM and possess an acceptable aselectivity against the other tested proteases. By contrast, their direct analogues with a neutral C-terminal 4-piperidyl-butanoyl methylamide residue have a reduced matriptase affinity and selectivity. Several of these monobasic compounds were found to be even more potent thrombin inhibitors with K_i values around 1 nM.

To reduce the strong affinity to thrombin and maintain high affinity as matriptase inhibitors, a second inhibitor series was prepared (Table 3.2). These derivatives contain a substituted urea residue in the C-terminal amide moiety combined with an N-terminal *β*-alanylamide group. Among this series, two matriptase inhibitors with K_i values below 10 nM could be identified. These analogues, containing a C-terminal cyclohexyl urea or methyl urea portions, were further optimized by replacement of their N-terminal basic *β*-alanylamide moiety with the most suitable 2,4-dimethoxyphenyl or 2,4-dichlorophenyl groups identified from the initial series. This provided the first monobasic matriptase inhibitor **241**, possessing an N-terminal 2′,4′-dimethoxybiphenylsulfonyl group and a C-terminal cyclohexylurea, which retains excellent matriptase affinity with an inhibition constant of 2.7 nM exhibiting negligible affinity to thrombin. This compound should be well suited to be used in further cell culture or animal studies,

probably also in a prodrug form, which are meanwhile well established for various benzamidino-drugs.

The relatively selective and potent matriptase inhibitors **241** and its analogues 2′,4′-dichlorobiphenyl **242**, as well as the dibasic inhibitor **224**, were examined for their inhibitory effect on the propagation of H9N2 influenza virus strains in MDCK II cells. All selected inhibitors could strongly suppress the multicycle virus replication at a concentration of 50 μM. The strongest inhibition is found for derivative **241**, which also showed a strong inhibitory effect at reduced concentrations of 10 μM and 20 μM. These results demonstrate that the tested matriptase inhibitors are effective in suppressing the replication of H9N2 influenza viruses in a matriptase-expressing MDCK II cell model. These matriptase inhibitors could be potential lead structures for the development of new drugs against H9 for influenza.

4.2 Modification of the 3-amidinophenylalanine in P1 position

All inhibitors of this series have strong reduced affinity to matriptase compared to the 3-amidinophenylalannine analogues. The incorporation of an arginine in inhibitor **247** (H-βAla-3-NH-phenylsulfonyl-Arg-4-(piperidin-4-yl)-*N*-methylbutanamide) reduced the potency by approximately 100-fold. All other modifications, e.g., the incorporation of propargylglycine-5-(pyridin-3-yl) and propargylglycine-5-(6-aminopyridin-3-yl) residues (**248** and **249)** have completely eliminated any matriptase affinity.

4.3 D-Phenylglycine derivatives

Additional inhibitors with a central D-phenylglycine residue, which were previously described as potent fXa inhibitors, were prepared and evaluated as matriptase inhibitors (section 3.3, Table 3.5). These derivatives differ in their substituted piperazine residue, which most likely occupies the S3/4 binding site of matriptase, whereas a constant 3-amidinobenzoyl-D-phenylglycyl segment was maintained.

The strongest matriptase inhibiton was found for the 4-pyridinyl-piperazine inhibitor **259** with a K_i value of 0.29 μM. The replacement of the terminal pyridine by various hydrophobic rings, such as benzyl, phenyl and 4-cyanophenyl led to more than 50-fold reduced matriptase inhibition ($K_i \geq 18$ μM). All of these D-phenylglycine derivatives reveal a weak potency against thrombin, and as expected from literatures possess a strong fXa affinity. For instance, compound **251** containing an N-terminal

4-cyanophenyl-piperazine inhibits fXa with a K_i value of 1 nM. The incorporation of a chlorothiophene carbonyl residue in P1 position, which is suitable for fXa inhibitors, resulted in a vompound with poor matriptase affinity.

4.4 Antranilamide derivatives

Based on some structural similarity between matriptase and uPA, both are Ser190 proteases, a few matriptase inihibitors, with a para-(guanidinomethyl)benzoyl aniline group as a potential P1 residue, were prepared. These compounds were derived from uPA inhibitors, like substituted anilides of 4-(2-aminoethoxy)benzoic acid or amiloride, which possesses an acylated guanidine as P1 residue. The obtained inhibitors **266**-**268** have only weak potency against matriptase and all other tested trypsin-like serine proteases. Therefore, no further derivatives were prepared in this series.

4.5 Substrate analogue inhibitors

Substrate analogue inhibitors, containing a C-terminal 4-amidinobenzylamide as P1 residue; a P4 benzylsulfonyl residue; and a P3 amino acid in D-configuration possess high potency against various trypsin-like serine proteases. During previous selectivity studies with such substrate analogue inhibitors of fXa, a few examples containing D-homophenylalanine derivatives in P3 positions were identified as moderate matriptase inhibitors. Therefore, a further screen of substrate analogue inhibitors available in our group was performed (section 3.5, Table 3.7).

The strongest affinity was found for Bzls-D-hPhe-Pro-4-amidinobenzylamide (**MI-0320**), which inhibits matriptase with a K_i value of 13 nM. Among a related series containing a constant P3 D-arginine residue, a relatively high potency was found for analogues with P2 alanine (**MI-0371**) or arginine (**MI-0360**). Both compounds inhibit matriptase with K_i values around 20 nM. The use of a P2 alanine, instead of proline, reduced the affinity to thrombin but maintained high potency against fXa. For instance, the alanine analogue **MI-0371** and the homoalanyl derivative **MI-0381** inhibit fXa with K_i values $\leq$ 2 nM. Other P2 residues were less suitable for matriptase inhibition. Additional analogues, missing the N-terminal benzylsulfonyl group were prepared to reduce their potency against fXa (section 3.5, Table 3.8).

This provided inhibitor **278** (H-D-hTyr-Ala-4-Amba), which inhibits matriptase with a K_i value of 26 nM, whereas it has more than 10 fold reduced potency against thrombin

and fXa. This compound is the first substrate analogue inhibitor with improved affinity to matriptase compared to other trypsin-like serine proteases. There also exist a few examples of thrombin inhibitors possessing a P3 amino acid in L-configuration. Therefore, a few analogues were prepared, which contain a Cα-dialkylated amino acid in P3 position. Such residues can be considered as a chimera between a D- and L-amino acid. However, the incorporation of these Cα-dialkylated amino acid residues in P3 position provided only poor matriptase inhibitors. The synthesis of these derivatives was much more complicated than the preparation of the classical substrate analogues containing D-amino acid as P3 residue.

Therefore, such dialkylated derivatives provide no further opportunity for the design of improved matriptase inhibitors.

4.6 Summary

Several new inhibitors were prepared and tested as potential matriptase inhibitors. The most potent compounds were found among the tertiary amides of arylsulfonylated-3-amidinophenylalanines. The incorporation of suitable bis-substituted biphenylsulfonyl groups in combination with appropriate C-terminal urea structures provided the final strong potent monobasic matriptase inhibitors with acceptable selectivity against related trypsin-like serine proteases. Such compounds are suitable candidates for their further evaluation in cell culture experiments to elucidate the potential role of matriptase in pathophysiological processes.

4.7 Zusammenfassung und Ausblick

Wirksame und selektive Matriptaseinhibitoren sind potentielle Wirkstoffe für die Behandlung verschiedener Krankheiten, wie Krebs und Metastasierung, Arthritis, Arteriosklerose oder durch H9-Influenzaviren verursachte Grippe. In der vorliegenden Arbeit wurden neue Matriptaseinhibitoren entwickelt und gegen verschiedene trypsinartige Serinproteasen, wie Matriptase, Thrombin und fXa getestet. Einige besonders wirksame Inhibitoren wurden zusätzlich noch auf ihre Hemmwirkung bezüglich anderer Proteasen, wie uPA, Matriptase-2 und HAT untersucht.

3-Amidinophenylalanin Derivate

Zunächst wurde eine neue Serie an Matriptaseinhibitoren synthetisiert, die sich vom 3-Amidinophenylalanin ableiten. Die wirksamsten Hemmstoffe dieses Typs besitzen eine N-terminale 2′,4′-Dimethoxy- oder 2′,4′-Dichlorbiphenyl-3-sulfonyl-Gruppe und einen C-terminalen 2-Aminoethylpiperidid-Rest. Diese bisbasische Verbindungen hemmen Matriptase mit K_i-Werten $\leq$ 2 nM und besitzen eine akzeptable Selektivität gegen andere trypsinartiger Serinproteasen. Im Gegensatz dazu besitzen die Analoga mit einem neutralen 4-Piperidyl-butanoylmethylamid-Rest am C-Terminus reduzierte Matriptaseaffinität und Selektivität. Einige dieser Verbindungen erwiesen sich mit K_i Werten um 1 nM sogar als deutlich stärkere Thrombininhibitoren.

Um die Matriptaseaffinität und Selektivität zu verbessern, wurde eine zweite Serie innerhalb dieses Strukturtyps synthetisiert. Diese Verbindungen besitzen am C-terminalen Piperididrest substituierte Harnstoffgruppen und am N-Terminus einen *ß*-Alanylamid-Rest. Unter diesen Verbindungen wurden zwei Matriptasehemmstoffe mit K_i-Werten unter 10 nM identifiziert, die C-terminal mit einem Cyclohexyl- oder Methylharnstoff-Rest modifiziert sind. Um monobasische Inhibitoren zu erhalten, die möglicherweise eine bessere Bioverfügbarkeit im Vergleich zu den dibasischen Verbindungen besitzen, wurde der N-terminale *ß*-Alanylamid-Rest durch 2,4-Dimeth-oxyphenyl- oder 2,4-Dichlorphenyl-Gruppen ausgetauscht, die sich bereits in der ersten Serie als besonders wirksam erwiesen hatten. Daraus resultierte der erste monobasische Matriptaseinhibitor **241**, der eine N-terminale 2′,4′-Dimeth-oxybiphenylsulfonyl-Gruppe und einen Cyclohexylharnstoff-Rest am C-Terminus besitzt. Diese Verbindung inhibiert Matriptase mit einer Hemmkonstanten von 2.7 nM und hat durch die sterisch

anspruchsvolle Cycloheyxylharnstoffgruppe vernachlässigbare Affinität zu Thrombin. Dieser Matriptaseinhibitor ist ein geeigneter Kandidat für weiterführende *in vitro* oder *in vivo* Tests, um die biologische Funktion dieser Protease genauer zu untersuchen und um pathogene Mechanismen zu hemmen, an denen Matriptase beteiligt ist. So wurde beispielsweise der monobasische Matriptaseinhibitor **241**, dessen direktes 2,4-Dichlorphenyl-Analogon **242**, sowie der dibasische Inhibitor **224** eingesetzt, um die Vermehrung der H9N2 Influenzaviren in MDCK II Zellen zu inhibieren. Alle drei Verbindungen besitzen bei einer Konzentration von 50 μM eine sehr starke Hemmwirkung auf die multizyklische Virusvermehrung, wobei Verbindung **241** auch bei niedrigeren Konzentrationen von 20 und 10 μM noch einen deutlichen Effekt zeigt. Demzufolge könnten diese Verbindungen eine potentielle Leitstruktur für die Behandlung von Grippeinfektionen durch H9-Viren darstellung. Denkbar ist auch eine Überführung dieser Inhibitoren in ein Prodrug, z.B. in eine Hydroxyamidino-Verbindung. Es ist bekannt, dass solche Prodrugs häufig eine deutlich bessere Bioverfügbarleit besitzen. Im Gegensatz zu den klassischen antiviralen Targets würden in diesem Fall stabile humane Zielstrukturen adressiert werden. Es ist davon auszugehen, dass sich in diesem Fall keine Wirkstoffresistenzen ausbilden würden. Diese Verbindungen könnten prinzipiell auch für eine Kombinationstherapie geeignet sein.

Austausch des 3-Amidophenylalanins in P1-Position

In einer weiteren Serie wurde das 3-Amidinophenylalanin in P1-Position durch andere Aminosäurereste ausgetauscht, jedoch zeigten alle erhaltenen Verbindungen eine reduzierte Matriptaseaffinität. Der Einbau eines Arginin, beispielsweise in Inhibitor **247** (H-βAla-3-NH-phenylsulfonyl-Arg-4-(Pip)-*N*-methylbutanamide), bewirkte eine ca. 100-fach geringere Hemmwirkung. Alle weiteren Austausche, wie der Einbau eines P1 3-Pyr-Propargylglycins in Inhibitor **248** oder des 4-Amino-3-Pyr-Propargylglycins in Hemmstoff **249** führten zu völlig wirkungslosen Verbindungen.

D-Phenylglycin-Derivate

Verbindungen mit einem zentralen D-Phenylglycin-Rest sind als wirksame fXa-Hemmstoffe aus der Literatur bekannt. Deshalb wurden analoge Verbindungen synthetisiert und erstmals als Matriptaseinhibitoren untersucht (Abschitt 3.3, Tabelle

3.5). Die hergestellten Verbindungen besitzen ein konstantes 3-Amidinobenzoyl-D-Phenylglycin-Segment und unterscheiden sich im substituierten Piperazin-Rest, der wahrscheinlich die S3/4-Tasche der Matriptase besetzt. Die stärkste Matriptasehemmung mit einem K_i-Wert von 0.29 µM wurde wurde für das 4-Pyridinyl-Piperazin Derivat **259** gefunden. Der Austausch des endständigen Pyridins durch hydrophobe Gruppen, wie Benzyl-, Phenyl- oder 4-Cyanophenyl führte zu einem mehr als 50-fachen Abfall der Hemmwirkung ($K_i \geq 18$ μM). Diese Verbindungen erwiesen sich auch als sehr schwache Thrombininhibitoren, sind jedoch sehr wirksame fXa-Hemmstoffe, wie aus der Literatur zu erwarten war. Beispielsweise hemmt die Verbindung **251** mit einem N-terminalen 4-Cyanophenyl-piperazin-Rest fXa mit einem K_i Wert von 1 nM.

Der aus der Entwicklung von fXa-Hemmstoffen dieses Strukturtyps bekannte Einbau eines Chlorthiophencarbonsäure-Restes in P1-Position führte zu drastischem Affinitätsverlust auf Matriptase.

Anthranilamid-Derivate

Vor kurzem wurden uPA-Hemmstoffe beschrieben, die eine p-Aminoethyloxybenzoyl-Gruppe als P1-Rest besitzen. Außerdem ist bekannt, dass uPA auch von dem Diuretikum Amilorid gehemmt wird und das dessen acyliertes Guanidin in die S1-Tasche der Protease bindet. Aufgrund struktureller Ähnlichkeiten in der S1-Tasche zwischen Matriptase und uPA, beide enthalten einen Ser190-Rest, wurden einige Verbindungen mit einem ähnlichen p-Aminoethyloxybenzoyl-Rest synthetisiert. Die hergestellten Inhibitoren **266**-**268** zeigten jedoch nur eine sehr schwache Matriptasehemmung und auch vernachlässigbare Affinität bezüglich anderer trypsinartiger Serinproteasen. Daher wurden in dieser Serie keine weiteren Derivate hergestellt.

Substratanaloge Inhibitoren

Substratanaloge Inhibitoren mit einem 4-Amidinobenzylamid in P1-Position und einem N-terminalen Benzylsulfonylrest sind sehr wirksame Inhibitoren vieler trypsinartiger Serinproteasen, wenn sie mit einem D-Aminosäurerest in P3-Position kombiniert werden. Frühere Selektivitätsstudien mit substratanalogen fXa-Inhibitoren zeigten, dass einige Verbindungen mit D-Homophenylalanin-Derivaten in P3-Position auch

Matriptase mit Hemmkonstanten ≥ 70 nM inhibieren. Daher wurden vorhandene Substratanaloga aus unserem Arbeitskreis als potentielle Matriptaseinhibition getestet (Abschnitt 3.5, Tabelle 3.7).

Die stärkste Affinität mit einem K_i-Wert von 13 nM wurde für die Verbindung Bzls-D-hPhe-Pro-4-Amba (**MI-0320**) gefunden. Ähnliche Derivate mit einem D-Arginin in P3-Position und Alanin (**MI-0371**) oder Arginin (**MI-0360**) als P2-Reste erwiesen sich mit Hemmkonstanten um 20 nM ebenfalls als relativ potente Matriptasehemmstoffe.

Der Austausch des P2-Prolins durch Alanin hatte kaum Einfluß auf die fXa-Affinität, bewirkte aber eine deutliche Reduktion der Hemmwirkung auf Thrombin. So hemmt beispielsweise das P2-Alaninanalogon **MI-0371** mit einem D-Arginin in P3-Position fXa mit einem K_i Wert von 1 nM. Andere P2-Reste eignen sich weniger für substratanaloge Matriptaseinhibitoren.

Um die fXa-Affinität zu reduzieren, wurde eine Serie von Verbindungen ohne N-terminalen Benzylsulfonyl-Rest synthetisiert (Abschnitt 3..5, Tabelle 3.8). Daraus resultierte der wirksame Matriptaseinhibitor **278** (H-D-hTyr-Ala-4-Amba, K_i 26 nM), der Thrombin und fXa Affinität deutlich schwächer mit Hemmkonstanten ≥ 300 nM hemmt. Diese Verbindung ist der erste substratanaloge Inhibitor, der Matriptase stärker als trypsinartige Serinproteasen hemmt.

Da auch Thrombininhibitoren mit L-Aminosäuren als P3-Reste bekannt sind, wurden einige Analoga mit Cα-dialkylierten Aminosäuren an dieser Position hergestellt. Derartige Aminosäuren können als Chimere zwischen D- und L-Aminosäuren angesehen werden. Die Synthese und Kopplung dieser Derivate erwies sich jedoch als sehr problematisch. Da alle Verbindungen aus dieser Serie nur eine moderate Matriptasehemmung zeigten, sind solche Hemmstoffe mit dialkylierten P3-Aminosäure keine günstigen Startstrukturen für die Entwicklung substratanaloger Matriptaseinhibitoren.

Zusammenfassung

Neben dem Screening vorhandener Verbindungen wurden zahlreiche neue Proteasehemmstoffe synthetisiert und als Matriptaseinhibitoren untersucht. Die wirksamsten Verbindungen sind tertiäre Amide arylsulfonylierter 3-Amidinophenyl-alanin-Derivate. Der Einbau geeigneter disubstituierter Biphenylsulfonyl-Reste in

Kombination mit substituierten C-terminalen Harnstoffen an der Piperididgruppe führte zu hochaffinen Matriptaseinhibitoren mit einem relativ günstigen Selektivitätsprofil gegenüber anderen trypsinartigen Serinproteasen. Diese Inhibitoren sind geeignete Kandidaten für weiterführende Experimente in Zellkultur oder zur Anwendung in Tiermodellen, um pathophysiologische Prozesse zu hemmen, die durch Matriptase vermittelt werden.

5 Experimental part

5.1 Materials and methods

5.1.1 Reagents and used materials

Standard chemicals and solvents were obtained from the companies Acros, VWR, Fisher Scientific, Fluka, Sigma-Aldrich, Merck, or Roth. The used solvents had p.a or HPLC-grade quality. Dry solvents were stored over molecular sieves. The acetonitrile for analytical and preparative HPLC was purchased from VWR (HiPerSolv CHROMANORM) or Sigma-Aldrich (CHROMASOLV for HPLC). Ultra pure water was prepared using a NOWA pure select system (KSN Water Technology, Nistertal). Aqueous solutions of acids, bases or salts were prepared using demineralized water. Trifluoroacetic acid was a gift from Solvay. Amino acids and their derivatives were purchased from Bachem, Novabiochem, Orpegen Pharma or IRIS Biotech.

5.1.2 Thin Layer Chromatography

Reaction control and product purity were monitored by using thin layer chromatography (TLC) on precoated "Aluminium oxide 60 F254" plates from Merck. A mixture of n-butanol/acetic acid/water (4:1:1, v/v/v) was used as a mobile phase. The compounds were visualized either by fluorescence detection (λ_{ex} 254 nm), or by spraying with a ninhydrin solution and heating (300 mg ninhydrin dissolved in 100 mL n-butanol and 3 mL glacial HOAc) followed by incubation in a chlorine gas atmosphere and spraying with an o-toluidine solution (150 mg o-toluidine and 2.1 g KI dissolved in 2 mL HOAc and 148 mL water and filtered) to reveal the spots. The calculated R_f -values describe the relationship between the migration distance of the sample to the solvent front from the initial spotting site.

5.1.3 HPLC

For all HPLC experiments the following solvents were used:

- Solvent A: 0.1% TFA in ultrapure water
- Solvent B: 0.1% TFA in ACN

Detection at 220 nm.

HPLC 1:
Unless otherwise mentioned, all analytical reversed-phase HPLC chromatograms were performed using a Shimadzu LC-10A gradient HPLC system consisting of the subsystems CTO-10A column oven, LC-10ATvp pumps (2 x), DGU-14A degasser, SIL-10Axl autoinjector, SCL-10Avp system controller, SPD-M 10Avp photodiode array detector, Shimadzu Class-VP software and a reversed phase column (Nucleodur

100-5 C_{18} ec, 250 mm x 4.6 mm, Macherey-Nagel, Düren, Germany). The detection was performed at 220 nm. Solvents A and B served as eluents at a flow rate of 1 mL/min and a linear gradient of 1 % increase in solvent B/min. Different starting conditions (1, 10, 20, 30 and 40% B) were used for analytical HPLC depending on the properties of the compounds, which are indicated for each derivative. The given purity for all intermediates is based on HPLC detection at 220 nm.

HPLC 2:
A few analytical HPLC-chromatograms for reaction monitoring and characterization of intermediates were performed using a Shimadzu LC-10A gradient HPLC system consisting of the subsystems CTO-10A column oven, LC-10AS pumps(2 x), SCL-10Avp system controller, SPD-M10A UV-Vis-detector, and a column for fast gradient applications (Chromolith® Performance 2 μM C-18 endcapped column, 130 Å, 100 mm × 4.6 mm from Merck). The detection was performed at 220 nm. Solvent A and solvent B served as eluents at a flow rate of 4 mL/min starting with 1% B and using a linear gradient of 10% increase in solvent B/min. In order to distinguish the data in the described experiments, this system is named as HPLC 2.

Preparative HPLC
The final inhibitors were purified using a preparative HPLC system (pumps: Varian PrepStar Model 218 gradient system, detector: ProStar Model 320, fraction collector: Varian Model 701). The used columns (of the dimensions 250 mm × 32 mm) were:

1. Nucleodur 100-5 C8ec, 100 Å, Macherey-Nagel, Düren
2. Nucleosil, 300-5 C18, 300 Å, Macherey-Nagel, Düren
3. Prontosil 120-5-C18-SH, 120 Å, Bischoff Chromatography

All purifications were performed using a linear gradient of acetonitrile/water containing 0.1 % TFA at a flow rate of 20 mL/min.

5.1.4 Lyophilization

All final inhibitors and some intermediates were obtained as TFA salts after preparative HPLC followed by lyophilization from water or 80% *tert*-butanol using a vacuo freeze-drying system Alpha 2-4 LD plus (Christ, Osterode am Harz, Germany).

5.1.5 Mass spectrometry

The molecular mass of the synthesized compounds was determined by a QTrap 2000 ESI mass spectrometry (Applied Biosystems, USA) or a VG Autospec (Micromass, USA).

5.2 Enzyme kinetic measurements

General procedures

The determination of the inhibition constants (K_i) of the synthesized inhibitors for various enzymes was performed using synthetic chromogenic p-nitroanilide substrates as described previously.[176] The optical density of the substrate cleavage was measured at 405 nm using a microplate IEMS Reader MF 1401 (Labsystems, Helsinki, Finland).
The used substrates were synthesized in our laboratory. These substrates were dissolved in ultrapure water. Stock solutions of the synthesized inhibitors were prepared with a concentration of 10 mM in DMSO and Tris·HCl buffer pH 8.0 (containing 0.154 M NaCl, 2 % ethanol) to prepare the appropriate concentrations. The used concentrations of the inhibitors in the assay were at least 10-fold higher than the enzyme concentration. For the calculation of the molecular weight of the inhibitors, one TFA molecule was added for each free basic group. The measurements were performed at RT with the following volumina:

- 200 µL 50 mM Tris·HCl buffer pH 8.0 (containing 0.154 M NaCl, 2 % ethanol and inhibitor in appropriate concentrations).
- 25 µL aqueous substrate solution
- start of reaction with 50 µL enzyme solution

The measurements were stopped with the addition of 25 μL 50% HOAc after an appropriate reaction time, when the absorbance at the highest substrate concentration in the absence of an inhibitor had reached a value between 0.15 and 0.18.[176] The K_i values were calculated according to the method of Dixon[236] at two different substrate concentrations and five different inhibitor concentrations using an EXCEL template developed by Stürzebecher. The K_i values are the mean of at least two independent measurements.
The following trypsine like serine proteases and respective substrates were used for the measurements (Table 5.1).

Table 5.1: Used enzymes and substrates.

Enzyme **stock concentration (assay concentration)**	**Substrate** **stock concentration, (assay concentration)**
matriptase[a] (catalytic domain)[78] 1.44×10^{-9} M (2.62×10^{-10} M)	$MeSO_2$-D-Cha-Gly-Arg-pNA; 2 mM (182 µM in assay) 1 mM (91 µM in assay) 0.5 mM (45.5 µM in assay)
human factor Xa (fXa)[a] 200.35 IE/mg, Enzyme Research South Bend 2.72×10^{-9} M (4.94×10^{-10} M)	CH_3OCO-D-Cha-Gly-Arg-pNA, 2 mM (182 µM in assay) 1 mM (91 µM in assay) 0.5 mM (45.5 µM in assay)
bovine thrombin[a][237] 1425 IE/mg 3.725×10^{-9} M (6.77×10^{-10} M)	$MeSO_2$-D-Cha-Gly-Arg-pNA; 2 mM (182 µM in assay) 1 mM (91 µM in assay) 0.5 mM (45.5 µM in assay)
human urokinase (uPA) [b] (HS medac) 0.06 mg/mL, 240 IE/mL Enzyme	Bz-ßAla-Gly-Arg-pNA as TFA salt 2 mM (182 µM in assay) 1 mM (91 µM in assay) 0.5 mM (45.5 µM in assay)

[a]Matriptase, fXa and thrombin stock solutions were prepared in 0.9% NaCl solution containing 0.1% HSA or BSA. [b]uPA stock solution was prepared in 0.9% NaCl solution.

5.3 General synthetic procedures

The following standard procedures A-S were performed as described previously in several textbooks[238, 239] for the synthesis of precursors, intermediates, and inhibitors.

Method A
Introduction of Boc protecting group[238, 240][5]

1 equiv of the amino component was dissolved in a mixture of dioxane and water (2:1, v/v, 5 mL/mmol) at 0 °C. This mixture was treated with 1.1 equiv 1 N NaOH and 1.1 equiv di-*tert*-butyl dicarbonate. The pH was adjusted to 8.5-9.5 with 1 N NaOH. The mixture was stirred for 3 h at RT, whereby the reaction was controlled by HPLC or TLC. If necessary, an additional amount of $(Boc)_2O$ and 1 N NaOH were added and the mixture was further stirred at RT overnight. The solvent was removed *in vacuo*. The residue was dissolved in EtOAc, washed trice with 5% $KHSO_4$-solution, and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered and removed *in vacuo*.

Method B
Cleavage of Boc-protecting group by 90% TFA[241][6]

1 equiv of Boc-protected compound was dissolved in 90% TFA (5 mL/mmol) and the mixture was stirred for 1 to 2 h at RT. The solvent was removed *in vacuo*. The product was purified by preparative RP-HPLC. Solvents were removed *in vacuo* and the product was lyophilized from water or 80% *tert*-butanol.

Method C
Cleavage of Boc-protecting group by 1 N HCl/acetic acid[241][7]

1 equiv of Boc-protected compound was dissolved in glacial HOAc (1 mL/mmol) and treated with 1 N HCl in glacial HOAc (5 mL/mmol). The mixture was shaken several times for 1-2 h at RT. The solvent was partially removed *in vacuo* and the product was precipitated by the addition of diethyl ether, filtered, washed with diethyl ether, and dried *in vacuo*.

Method D
Introduction of Cbz protectining group[238][8]

1 equiv of the amino derivative was dissolved in ACN (10 mL/mmol) and treated with 2 equiv NMM. 1.1 equiv Cbz-OSu dissolved in ACN (5 mL/mmol) were added in two

[5] Page 90 of Ref. 240

[6] Page 170

[7] Page 169

[8] Page 52

portions and the mixture was stirred at 0 °C for 1h. The pH value was set to 8.5-9.5 by the addition of NMM and the mixture was stirred at RT for 3 h (HPLC control). If necessary, an additional amount of Cbz-OSu and NMM were added and the mixture was further stirred at RT overnight. The solvent was removed *in vacuo*. The residue was dissolved in EtOAc and washed trice with 5% $KHSO_4$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

Method E

Cleavage of Cbz-protecting group by HBr/acetic acid[241]₉

1 equiv of Cbz-protected compound was dissolved in 32% HBr in glacial HOAc (10 mL/mmol).The mixture was shaken several times within 1 h at RT. The solvent was partially removed *in vacuo* and the product was precipitated by diethyl ether, filtered, washed with diethyl ether, and dried *in vacuo*. Alternatively, the product was purified by preparative RP-HPLC, and lyophilized from water or 80% *tert*-butanol.

Method F

Hydrogenation[241]₁₀

The compound was dissolved in 90% HOAc and treated with approximately 10 mass percent of catalyst (10% Pd/C). The mixture was stirred at least overnight in a hydrogen atmosphere. If necessary, the hydrogenation was continued at RT for additional 24 h. The catalyst was removed by filtration and the solvent was evaporated. The product was purified by preparative HPLC and lyophilized from water or 80% *tert*-butanol.

Method G

Introduction of the Tfa-group[238]₁₁

1 equiv of the amino component was dissolved in 2 equiv TFA and treated with 2 equiv of trifluoroacetic anhydride. The mixture was stirred at 0 °C for 1 h and at RT for 2 h. The reaction progress was monitored by HPLC, and if necessary, an additional amount of trifluoroacetic anhydride was added and stirring was continued at RT overnight. The solvent was removed *in vacuo*. The remaining residue was dissolved in EtOAc and washed trice with 5% $KHSO_4$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.

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[10] Page 153-156

[11] Page 174

Method H

Cleavage of the Tfa-protecting group and saponification of methyl or ethyl esters with 1 N NaOH[241][12]

The corresponding ester or a Tfa-protected amino derivative was dissolved in a mixture of dioxane and water (1:1, 10 mL/mmol) and the pH was adjusted to 12 using 1 N NaOH. The mixture was stirred for 3 h (HPLC control), and if necessary, stirring was continued overnight at RT until the end of the reaction. The solution was neutralized by the addition of 10% TFA and the solvent was removed *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized from 80% *tert*-butanol.

Method I

PyBOP-coupling[242]

1 equiv of the salt of an amino component and 1 equiv of the carboxyl derivative were dissolved in DMF (5 mL/mmol) and treated with 1.05 equiv PyBOP and 3 equiv DIPEA. The mixture was stirred at 0 °C for 30 minutes and at RT for 2 h. The solvent was removed *in vacuo*. The product was purified using preparative HPLC. Alternatively, the residue was dissolved in EtOAc and washed trice with 5% $KHSO_4$-solution, once with brine, trice with saturated $NaHCO_3$-solution, and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

Method J

EDCI, HOBT-coupling[204, 243, 244]

1 equiv of the amino component and 1 equiv of the carboxyl component were dissolved in DMF (5 mL/mmol) and treated with 1.25 equiv EDCI and 1.1 equiv HOBT. The mixture was stirred under argon atmosphere at RT for 3 h. The solvent was removed *in vacuo*. The product was purified using preparative HPLC. Alternatively, the residue was dissloved in EtOAc and washed trice with 5% $KHSO_4$-solution, once with brine, trice with saturated $NaHCO_3$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The product was lyophilized from water or 80% *tert*-butanol.

Method K

HATU-coupling[238][13]

1 equiv of the salt of an amino component and 1 equiv of the carboxyl component were dissolved in DCM (5 mL/mmol) and treated with 1.25 equiv HATU and 2 equiv DIPEA. The mixture was stirred at 0 °C for 1 h. The pH value was maintained at 8-9 by the addition of DIPEA and the mixture was continued to be stirred at RT for 2 h. The

[12] Page 177

[13] Page 568

solvent was removed *in vacuo*. The residue was dissolved in EtOAc and washed trice with 5% $KHSO_4$-solution, once with brine, trice with saturated $NaHCO_3$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

Method L

Mixed anhydride procedure[78, 239][14]

1 equiv of the carboxyl component was dissolved in DMF (5 mL/mmol) at -15 °C and treated with 1 equiv NMM and 1 equiv isobutyl chloroformate. The mixture was stirred at -15 °C for 10 min. The mixed anhydride was treated with 1 equiv of the salt of an amino component and 1 equiv NMM. The mixture was stirred at -15 °C for 1 h and at RT overnight. The solvent was removed *in vacuo*. The residue was dissolved in EtOAc and washed trice with 5% $KHSO_4$-solution, once with brine, trice with saturated $NaHCO_3$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

Method M

Introduction of sulfonyl groups[229]

1 equiv of the amino acid was dissolved in dioxane and water or ACN and water (2:1, v/v, 15 mL/mmol). The pH was adjusted to 8-9 with ca. approximately 1 equiv DIPEA. 1.1 equiv sulfonyl chloride was added in several portions at 0 °C and the pH was maintained at 8-9 by the addition of DIPEA. The mixture was stirred at 0 °C for 1 h and at RT overnight. The solvent was removed *in vacuo*, the residue was dissolved in water and the pH was set to ca. 10 with 1 N NaOH. The water phase was extracted once with EtOAc and the pH was set to ca. 2 with 6 N HCl. The water phase was extracted trice with EtOAc. The combined EtOAc phase was washed trice with 5% $KHSO_4$-solution, trice with brine, dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.

Method N

Suzuki coupling procedure[171, 245]

1 equiv of the intermediates 3-bromophenylsulfonyl-Phe(3-CN)-4-(piperidin-4-yl)-*N*-methylbutanamide or 3-bromophenylsulfonyl-Phe(3-CN)-2-(piperidin-4-yl)Boc-ethyl-amine, 1.5 equiv arylboronic acid and 2 mL/mmol 2M Cs_2CO_3 were suspended in toluene (≈ 3 mL/mmol). In an argon atmosphere, 1 mol% Pd-(II)-acetate and 2 mol% 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl (S-Phos, Aldrich) were added and the mixture was heated under reflux for 3 h. The mixture was centrifuged and the solvent was removed from the supernatant *in vacuo*. The remaining residue was dissolved in EtOAc and washed trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

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Method O

Conversion of nitrile into hydroxyamidine[229]

1 equiv of the benzonitrile derivative was dissolved in absolute ethanol (5 mL/mmol), treated with 3 equiv hydroxylamine × HCl, and 3 equiv DIPEA. The mixtrure was heated under reflux for 3-4 hours and stirred at RT overnight. The solvent was removed *in vacuo* and the remaining residue was used for method P without further purification.

Method P

Acetylation of the hydroxyamidine[188]

1 equiv of the hydroxyamidine derivative was dissolved in glacial HOAc (10 mL/mmol), treated with 3 equiv acetic anhydride and stirred at RT for 1 h. The solvent was removed *in vacuo* (temperature of the water bath has to be < 35 °C to avoid 1,2,4-oxadiazole formation). The oily intermediate was used for the next step (method F or Q) without further purification.

Method Q

Reduction of acetylhydroxyamidine- or nitro- derivatives by zinc in acetic acid

1 equiv of the acetylhydroxyamidine or nitro derivative was dissolved in 90% HOAc (50 mL/mmol) and treated with zinc dust. The mixture was stirred at RT for 1-2 h (HPLC control). After filtration, the solvent was evaporated *in vacuo*, and the product was purified by preparative HPLC, followed by lyophilization from 80% *tert*-butanol. In the case of the reduction of nitro derivatives, the product was dissolved in EtOAc, washed trice with saturated $NaHCO_3$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

Method R

Preparation of urea derivatives[190, 191]

1 equiv of the free amino derivative was dissolved in DCM (15 mL/mmol), treated with 1.5 equiv isocyanate and 2 equiv TEA. The mixture was stirred at RT overnight. The solvent was removed *in vacuo*, the residue was dissolved in EtOAc, washed trice with saturated $NaHCO_3$-solution, and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

Method S

Conversion of amino into guanidino group[214]

1 equiv of the free amino intermediate was dissolved in DMF (10 mL/mmol), treated with 2 equiv 1-*H*-pyrazole-carboxamidine, and 2 equiv DIPEA. The mixture was stirred at RT for 24 h (HPLC control) and if necessary, an additional amount of 1-*H*-pyrazole-carboxamidine and DIPEA were added, and stirring was continued until completion of the reaction. The solvent was removed *in vacuo*, the product was purified by preparative RP-HPLC and lyophilized from water or 80% *tert*-butanol.

5.4 Synthesis of intermediates

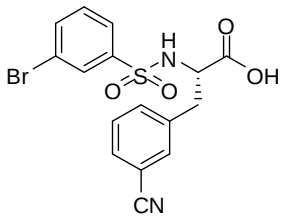
In this section, the synthesis of the intermediates is described. If needed, these syntheses were repeated with larger quantities. Due to the small scale of synthesis in many cases the obtained crude intermediates were directly used for the next step without purification to homogeneity. Their purity was characterized by HPLC detection at 220 nm and their yield is only provided in mg without conversion into the amount of mmol.

Compound names in bold were generated by Chemoffice Ultra 12.0 according to the IUPAC nomenclature. In the case of amino acid derivatives, additional shorter common names are provided in brackets. For simplification, the counter ions are omitted in the chemical structures.

5.4.1 Synthesis of 3-amidinophenylalanine derivatives

1) (*S*)-2-(3-bromophenylsulfonamido)-3-(3-cyanophenyl)propanoic acid

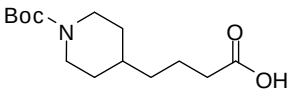
[3-Br-phenylsulfonyl-Phe(3-CN)-OH][171, 245]



675 mg (3.55 mmol) H-Phe(3-CN)-OH were dissolved in a mixture of dioxane and water and treated with 1 g (3.914 mmol) 3-bromophenylsulfonyl chloride according to method M.
Yield: 1.165 g (2.85 mmol, 80.2%), yellowish solid.
HPLC: 49.5% B (purity: 100%); MS calcd: 407.98, found: 407.09 $(M-H)^-$, 409.10 $(M+H)^+$.

2) 4-(1-(*tert*-butoxycarbonyl)piperidin-4-yl)butanoic acid

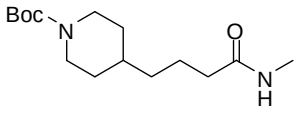
[4-(1-Boc-Pip)butanoic acid)][245]



4 g (19.3mmol) 4-piperidine butanoic acid × HCl were treated with di-*tert*-butyl dicarbonate according to method A.
Yield: 4.8 g (17.7 mmol), colourless crystals.
HPLC: 51.9% B (purity: 98%); TLC: R_f 0.78.

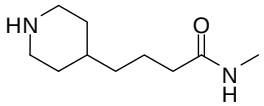
3) *tert*-butyl 4-(4-(methylamino)-4-oxobutyl)piperidine-1-carboxylate

[*N*-methyl-4-(Boc-Pip)butanamide][245]



1 g (3.69 mmol) intermediate **2** was dissolved in 10 mL EtOAc and treated with 2.5 g (36.9 mmol) methylamine × HCl, 2.1 g (5.54 mmol) HBTU and 2.89 mL (16.61 mmol) DIPEA. The mixture was stirred at 40 °C for 5 h. The solution was cooled to RT and washed trice with 5% $KHSO_4$-solution, once with brine, trice with saturated $NaHCO_3$-solution, trice with brine and dried over Na_2SO_4. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.
Yield: 884 mg, colourless oil.
HPLC: 46.6% B (purity: 95%).

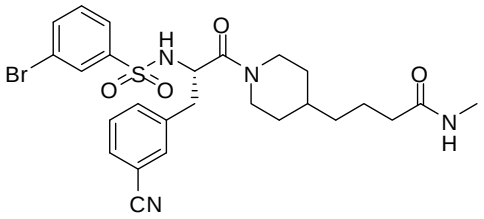
4) *N*-methyl-4-(piperidin-4-yl)butanamide × HCl[245]



884 mg of intermediate **3** were treated with 1 N HCl in HOAc according to method C.
Yield: 600 mg (2.71 mmol, 87.4%), white solid.
HPLC: 19.0% B (purity: 98%); MS calcd: 184.16, found: 185.10 $(M+H)^+$.

5) (*S*)-4-(1-(2-(3-bromophenylsulfonamido)-3-(3-cyanophenyl)propanoyl)piperidin-4-yl)-*N*-methylbutanamide

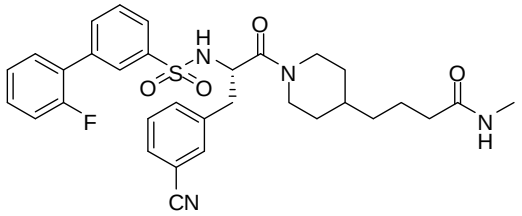
[3-Br-phenylsulfonyl-Phe(3-CN)-4-Pip-*N*-methylbutanamide][245]



550 mg (1.34 mmol) intermediate **1** was coupled to 326 mg (1.474 mmol) intermediate **4** according to method I.
Yield: 500 mg, yellow oil.
HPLC: 52.5% B (purity: 91%), HPLC 2: 46.1% B; MS calcd: 574.12, found: 575.09 $(M+H)^+$.

6) (*S*)-4-(1-(3-(3-cyanophenyl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)-propanoyl)-piperidin-4-yl)-*N*-methylbutanamide

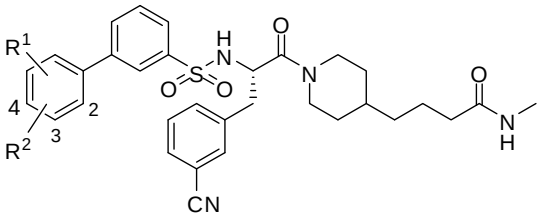
[2′F-biphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-*N*-methylbutanamide]



50 mg intermediate **5** were treated with 18.2 mg (0.13 mmol) 2-fluorophenylboronic acid according to method N.
Yield: 30 mg (0.051 mmol), yellow oil.
HPLC 2: 50.6% B (purity: 96.2 %), MS calcd: 590.24, found: 591.22 $(M+H)^+$.

Additional analogues **7-13** (Table 52) were prepared according to method N, using 50 mg intermediate **5** and 0.13 mmol (1.5 equiv.) of various substituted phenylboronic acids.

Table 5.2: Synthesis, yield, and analytical characterization of compounds **7-13** having the general formula.

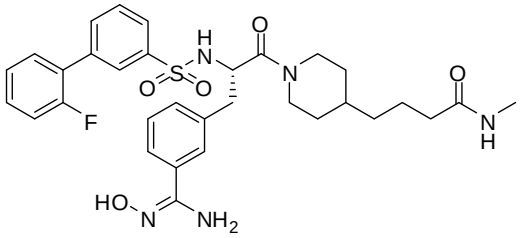


1	R^1	R^2	Amount boronic acid mg	Yield[a] mg/mmol	HPLC % B, purity %	MS calcd, found $(M+H)^+$
7	3-F	-	18.2	25/0.042	50.9[b], 95.9	590.24, 591.22
8	4-F	-	18.2	40/0.068	51.3[b], 96.3	590.24, 591.22
9	2-Cl	4-Cl	24.9	35/0.055	56.5[b], 60.1	640.17, 641.30
10	2-Cl	3-Cl	24.9	40/0.062	62.9, 71.1	640.17, 641.31
11	3-Cl	4-Cl	24.9	42/0.065	64.6, 72.4	640.17, n.d.
12	2-Cl	4-EtO	26.1	30/0.046	64.5, 64.8	650.23, n.d.
13	2-MeO	4-MeO	23.7	35/0.055	57.5, 90.3	632.27, n.d.

[a]yellow to brownish oil, [b]HPLC 2.

14) (*S*)-4-(1-(2-(2'-fluorobiphenyl-3-ylsulfonamido)-3-(3-(*N*'-hydroxycarbamimidoyl)phenyl)propanoyl)piperidin-4-yl)-*N*-methylbutanamide

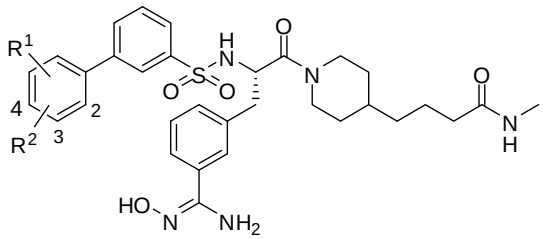
[2′F-biphenyl-3-sulfonyl-Phe(3-OxAm)-4-Pip-*N*-methylbutanamide]



30 mg of the intermediate **6** were converted into hydroxyamidine intermediate according to method O. The residue was directly used for the preparation of intermediate **22** without further purification.
Yield: not determined, yellow oil.
HPLC 2: 41.9% B (purity: 80.1 %).

Additional analogues **15-21** (Table 5.3) were prepared from intermediates **7-13** using method O.

Table 5.3: Synthesis, yield, and analytical characterization of compounds **15-21** having the general formula.

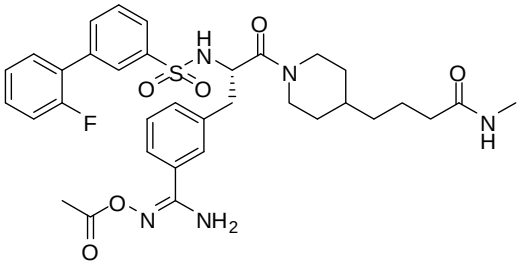


No.	R^1	R^2	Educt		HPLC
			Intermediate. No.	mg	% B, purity %
15	3-F	-	**7**	25	41.8[a], 77.9
16	4-F	-	**8**	40	42.5[a], 85.9
17	2-Cl	4-Cl	**9**	35	48.2, 50.3
18	2-Cl	3-Cl	**10**	40	48.1, 50.5
19	3-Cl	4-Cl	**11**	42	48.9, 74.2
20	2-Cl	4-EtO	**12**	30	50.7, 60.5
21	2-MeO	4-MeO	**13**	35	43.9, 42.9

[a]HPLC 2

22) (*S*)-4-(1-(3-(3-(*N*'-acetoxycarbamimidoyl)phenyl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-yl)-*N*-methylbutanamide

[2′F-biphenyl-3-sulfonyl-Phe(3-AcOxAm)-4-Pip-*N*-methylbutanamide]



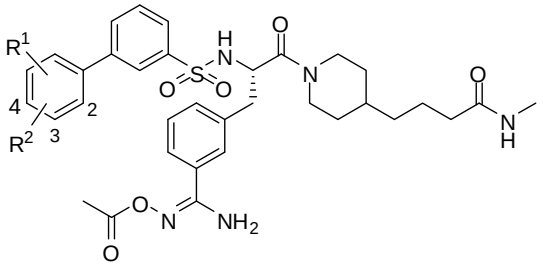
The intermediate **14** was acetylated according to method P. The solvent was removed *in vacuo* and the remaining oily crude was used for the synthesis of inhibitor **MI-0403** without further purification.

Yield: not determined, yellow oil.

HPLC 2: 46.9% B (purity: 78.5%).

Additional derivatives **23-29** (Table 5.4) were prepared by an analogous procedure from intermediates **15-21**.

Table 5.4: Synthesis, yield, and analytical characterization of compounds **23-29** having the general formula.

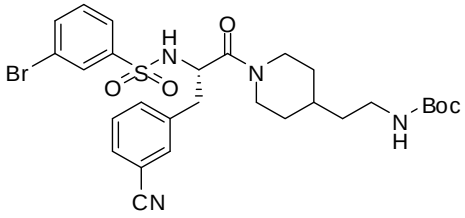


No.	R^1	R^2	Educt Intermediate No.	HPLC % B, purity %
23	3-F	H	**15**	47.6[a], 62.7
24	4-F	H	**16**	47.5[a], 82.2
25	2-Cl	4-Cl	**17**	53.5[a], 37.2
26	2-Cl	3-Cl	**18**	57.3, 36.1
27	3-Cl	4-Cl	**19**	58.6, 70.2
28	2-Cl	4-EtO	**20**	59.1, 50.1
29	2-MeO	4-MeO	**21**	52.3, 41.7

[*]HPLC 2

30) (*S*)-*tert*-butyl 2-(1-(2-(3-bromophenylsulfonamido)-3-(3-cyanophenyl)-propanoyl)piperidin-4-yl)ethylcarbamate

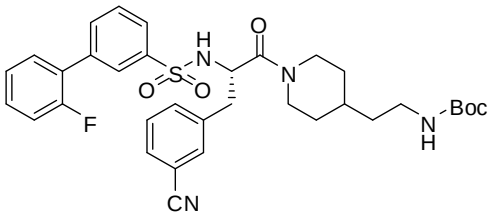
[3-Br-phenylsulfonyl-Phe(3-CN)-4-Pip-Boc-ethylamine][245]



800 mg (1.95 mmol) intermediate **1** were coupled to 491 mg (2.15 mmol) *tert*-butyl 2-(piperidin-4-yl)ethylcarbamate[78] according to method I.
Yield: 820 mg (≈ 1.323 mmol), yellow oil.
HPLC: 64.5% B (purity: 80.5%); MS calcd: 618.15, found: 619.19 $(M+H)^+$.

31) (*S*)-*tert*-butyl 2-(1-(3-(3-cyanophenyl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)-propanoyl)piperidin-4-yl)ethylcarbamate

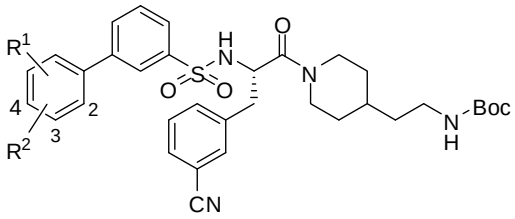
[2′F-biphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-Boc-ethylamine]



50 mg intermediate **30** were coupled with 17 mg (0.121 mmol) 2-fluorophenylboronic acid according to method N.
Yield: 35 mg (0.055 mmol), yellow oil.
HPLC: 68.2% B (purity: 90.9%).

Additional analogues **32-38** (Table 5.5) were prepared according to method N using 50 mg intermediate **30** and 0.121 mmol (1.5 equiv) of various substituted phenylboronic acids.

Table 5.5: Synthesis, yield, and analytical characterization of compounds **32-38** having the general formula.

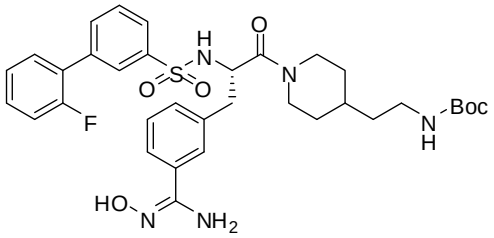


No.	R^1	R^2	Amount boronic acid mg	Yield[a] mg	HPLC % B, purity %	MS calcd, found $(M+H)^+$
32	3-F	-	17	38	68.5, 80.8	634.26, 635.30
33	4-F	-	17	35	68.2, 80.3	634.26, 635.30
34	2-Cl	4-Cl	23.2	40	74.6, 72.1	684.19, 685.12
35	2-Cl	3-Cl	23.2	36	74.3, 71.2	684.19, nd.
36	3-Cl	4-Cl	23.2	30	75.9, 76.2	684.19, 685.20
37	2-Cl	4-EtO	24.4	35	75.1, 97.4	694.26, nd.
38	2-MeO	4-MeO	22.1	38	69.5, 75.6	676.29, 677.41

[a]yellow to brownish oil

39) (*S*)-*tert*-butyl 2-(1-(2-(2'-fluorobiphenyl-3-ylsulfonamido)-3-(3-(*N*'-hydroxycarbamimidoyl)phenyl)propanoyl)piperidin-4-yl)ethylcarbamate

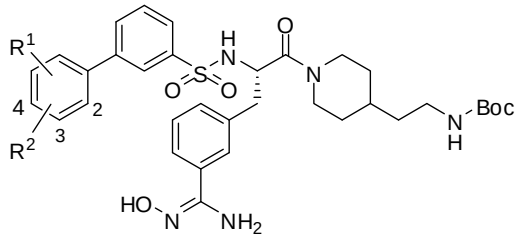
[2′F-biphenyl-3-sulfonyl-Phe(3-OxAm)-4-Pip-Boc-ethylamine]



35 mg of the nitrile intermediate **31** were converted into hydroxyamidine according to method O. The remaining oily residue was used for the preparation of intermediate **47** without further purification.
Yield: not determined, yellow oil.
HPLC: 51.2% B (purity: 75.2%).

Additional analogues **40-46** (Table 5.6) were prepared from intermediate **32-38** in analogy to compound **39** using method O.

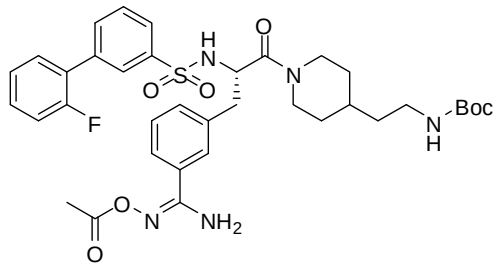
Table 5.6: Synthesis, yield, and analytical characterization of compounds **40**-**46** having the general formula.



No.	R^1	R^2	Educt		HPLC
			Intermediate. No.	mg	% B, purity %
40	3-F	-	**32**	38	51.3, 80.8
41	4-F	-	**33**	35	51.2, 81.9
42	2-Cl	4-Cl	**34**	40	58.2, 50.1
43	2-Cl	3-Cl	**35**	36	58.1, 50.8
44	3-Cl	4-Cl	**36**	30	58.5, 67.2
45	2-Cl	4-EtO	**37**	35	57.3, 55.1
46	2-MeO	4-MeO	**38**	38	52.2, 52.3

47) (*S*)-*tert*-butyl 2-(1-(3-(3-(*N*'-acetoxycarbamimidoyl)phenyl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-yl)ethylcarbamate

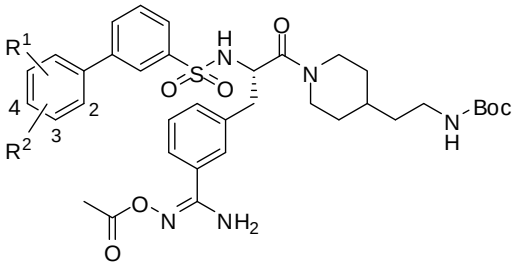
[2′F-biphenyl-3-sulfonyl-Phe(3-AcOxAm)-4-Pip-Boc-ethylamine]



The intermediate **39** was acetylated with acetic anhydride according to method P. The remaining residue was used for the synthesis of intermediate **55** without further purification.
Yield: not determined, yellow oil.
HPLC: 60% B (purity: 52.4%).

Additional analogues **48**-**54** (Table 5.7) were prepared by acetylation of intermediates **40**-**46** according to the method P.

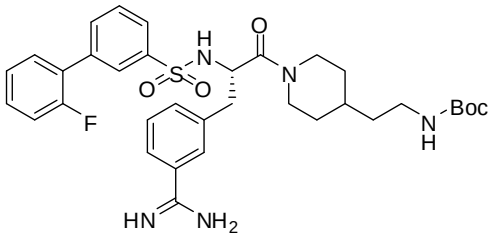
Table 5.7: Synthesis, yield, and analytical characterization of compounds **48-54** having the general formula.



No.	R^1	R^2	Educt Intermediate No.	HPLC % B, purity %
48	3-F	H	**40**	59.9, 54.9
49	4-F	H	**41**	60.1, 70.3
50	2-Cl	4-Cl	**42**	68.9, 69.1
51	2-Cl	3-Cl	**43**	68.3, 48.9
52	3-Cl	4-Cl	**44**	69.8, 60.1
53	2-Cl	4-EtO	**45**	63.7, 30.4
54	2-MeO	4-MeO	**46**	63.4, 60.1

55) (*S*)-*tert*-butyl 2-(1-(3-(3-carbamimidoylphenyl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-yl)ethylcarbamate

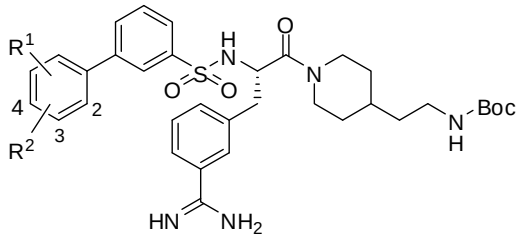
[2′F-biphenyl-3-sulfonyl-Phe(3-Am)-4-Pip-Boc-ethylamine]



The intermediate **47** was reduced by zinc dust in 90% HOAc according to method Q. The remaining oily residue was used without further purification for the synthesis of inhibitor **MI-0433**.
Yield: not determined, colourless oil.
HPLC: 51.4% B (purity: 76.7%).

Additional analogues **56-61** (Table 5.8) were prepared by reduction of the intermediates **48-53** according to method Q.

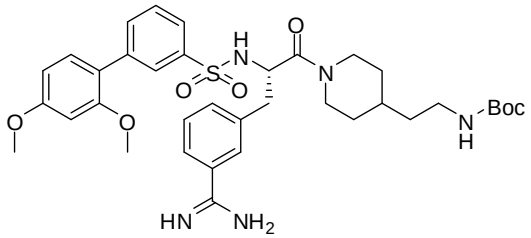
Table 4.5: Synthesis, yield and analytical characterization of compounds **56**-**61** having the general formula.



No.	R^1	R^2	Educt Intermediate No.	HPLC % B, purity %
56	3-F	H	**48**	51.3, 69.5
57	4-F	H	**49**	51.2, 67.2
58	2-Cl	4-Cl	**50**	58.2, 78.2
59	2-Cl	3-Cl	**51**	58.1, 66.8
60	3-Cl	4-Cl	**52**	58.5, 67.5
61	2-Cl	4-EtO	**53**	57.3, 49.9

62) (*S*)-*tert*-butyl 2-(1-(3-(3-carbamimidoylphenyl)-2-(2',4'-dimethoxybiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-yl)ethylcarbamate

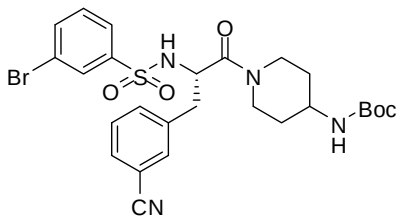
[2′,4′-dimethoxybiphenyl-3-sulfonyl-Phe(3-Am)-4-Pip-Boc-ethylamine]



The intermediate **54** was hydrogenated for 48 h according to method F. The catalyst was removed by filtration, the solvent was evaporated and the remaining oily residue was used for the preparation of inhibitor **MI-0439** without further purification.
Yield: not determined, colourless oil.
HPLC: 52.2% B (purity: 63.2%).

63) (*S*)-*tert*-butyl 1-(2-(3-bromophenylsulfonamido)-3-(3-cyanophenyl)-propanoyl)piperidin-4-ylcarbamate

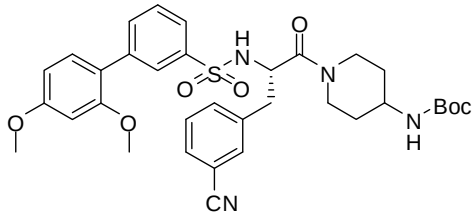
[3-bromophenylsulfonyl-Phe(3-CN)-4-Pip-NH-Boc][78]



200 mg (0.489 mmol) intermediate **1** were coupled to 127.3 mg (0.538 mmol) *tert*-butyl piperidin-4-ylcarbamate (Aldrich) according to method I.
Yield: 225 mg (≈ 0.38 mmol), yellowish solid.
HPLC: 62.6% B (purity: 92.3%); MS calcd: 590.1, found: 591.1 $(M+H)^+$.

64) (*S*)-*tert*-butyl 1-(3-(3-cyanophenyl)-2-(2',4'-dimethoxybiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-ylcarbamate

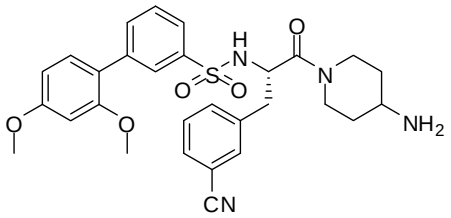
[2′,4′-dimethoxybiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-NH-Boc]



50 mg intermediate **63** were coupled with 23.1 mg (0.127 mmol) 2,4-dimethoxyphenylboronic acid according to method N.
Yield: 50 mg, yellow oil.
HPLC: 67.4% B (purity: 66.3%).

65) (*S*)-*N*-(1-(4-aminopiperidin-1-yl)-3-(3-cyanophenyl)-1-oxopropan-2-yl)-2',4'-dimethoxybiphenyl-3-sulfonamide

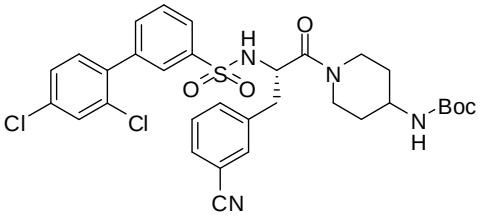
[2′,4′-dimethoxybiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-NH_2]



50 mg intermediate **64** were deprotected according to method B. The product was dissolved in EtOAc, washed twice with saturated $NaHCO_3$-solution and twice with brine. The EtOAc phase was dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.
Yield: 38 mg, oil.
HPLC: 44.5% B (purity: 72.3%).

66) (*S*)-*tert*-butyl 1-(3-(3-cyanophenyl)-2-(2',4'-dichlorobiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-ylcarbamate

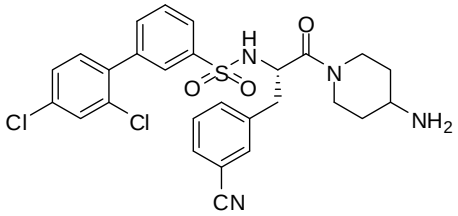
[2′,4′-dichlorobiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-NH-Boc]



50 mg (0.085 mmol) intermediate **63** were coupled with 24.2 mg (0.127 mmol) 2,4-dichlorophenylboronic acid according to method N.
Yield: 46 mg, yellowish oil.
HPLC: 74.5% B (purity: 68.8%).

67) (*S*)-*N*-(1-(4-aminopiperidin-1-yl)-3-(3-cyanophenyl)-1-oxopropan-2-yl)-2',4'-dichlorobiphenyl-3-sulfonamide

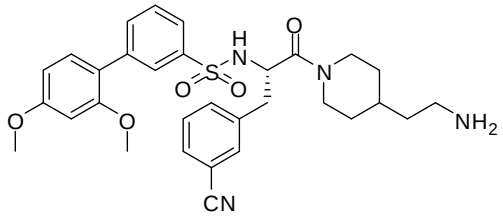
[2′,4′-Dichlorobiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-NH_2]



46 mg intermediate **66** were deprotected according to method B. The product was dissolved in EtOAc and washed twice with saturated $NaHCO_3$-solution and twice with brine. The EtOAc phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.
Yield: 38 mg, yellow oil.
HPLC: 49.9% B (purity: 90.2%).

68) (*S*)-*N*-(1-(4-(2-aminoethyl)piperidin-1-yl)-3-(3-cyanophenyl)-1-oxopropan-2-yl)-2',4'-dimethoxybiphenyl-3-sulfonamide

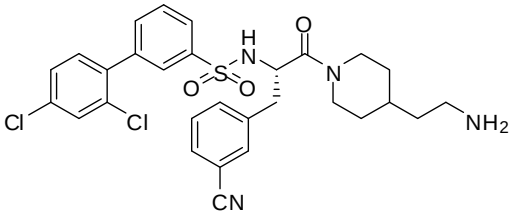
[2′,4′-dimethoxybiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-ethylamine]



50 mg intermediate **38** were deprotected according to method B. The product was dissolved in EtOAc, washed twice with saturated $NaHCO_3$-solution, twice with brine and dried over Na_2SO_4. The EtOAc phase was filtered and the solvent was removed *in vacuo*.
Yield: 42 mg, yellowish oil.
HPLC: 46.1% B (purity: 78.5%).

69) (*S*)-*N*-(1-(4-(2-aminoethyl)piperidin-1-yl)-3-(3-cyanophenyl)-1-oxopropan-2-yl)-2',4'-dichlorobiphenyl-3-sulfonamide

[2′,4′-dichlorobiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-ethylamine]

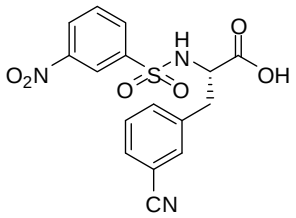


50 mg intermediate **34** were deprotected according to method B. The product was dissolved in EtOAc, washed twice with saturated $NaHCO_3$-solution, twice with brine

and dried over Na_2SO_4. The EtOAc phase was filtered and the solvent was removed *in vacuo*.
Yield: 40 mg, brownish oil.
HPLC: 50.9% B (purity: 79.5%).

70) (*S*)-3-(3-cyanophenyl)-2-(3-nitrophenylsulfonamido)propanoic acid

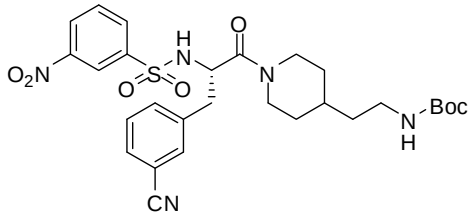
[3-NO_2-phenylsulfonyl-Phe(3-CN)-OH][78]



1.034 g (5.436 mmol) H-Phe(3-CN)-OH were dissolved in a mixture of ACN and water and treated with 1.325 g (5.980 mmol) 3-nitrophenylsulfonyl chloride according to method M, whereby 1 N NaOH (≈ 6mL) was used instead of DIPEA to set the pH to 8-9.
Yield: 1.246 g (3.319 mmol; 61.1%), yellowish solid.
HPLC: 45.9% B; MS calcd: 375.05, found: 374.09 $(M-H)^-$, 376.10 $(M+H)^+$.

71) (*S*)-*tert*-butyl 2-(1-(3-(3-cyanophenyl)-2-(3-nitrophenylsulfonamido)-propanoyl)piperidin-4-yl)ethylcarbamate

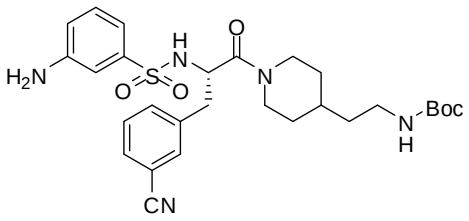
[3-NO_2-phenylsulfonyl-Phe(3-CN)-4-Pip-Boc-ethylamine][78]



1 g (2.664 mmol) intermediate **70** was coupled with 0.67 g (2.93 mmol) *tert*-butyl 2-(piperidin-4-yl)ethylcarbamate according to method I.
Yield: 1.225 g (2.09 mmol; 78.5%), orange solid.
HPLC: 61.4% B (purity: 96.5%); MS calcd: 585.23, found: 586.21 $(M+H)^+$.

72) (*S*)-*tert*-butyl 2-(1-(2-(3-aminophenylsulfonamido)-3-(3-cyanophenyl)-propanoyl)piperidin-4-yl)ethylcarbamate

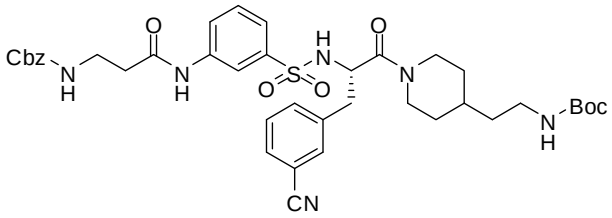
[3-NH_2-phenylsulfonyl-Phe(3-CN)-4-Pip-Boc-ethylamine][78]



1.225 g (2.09 mmol) intermediate **71** were reduced using zinc dust in 90% acetic cid according to method Q.
Yield: 1.05 g (1.889 mmol; 90.4%), orange solid.
HPLC: 51.9% B (purity: 95.5%); MS calcd: 555.25, found: 556.21 $(M+H)^+$.

73) Benzyl 3-amino-3-oxopropylcarbamate-(S)-*tert*-butyl 2-(1-(2-(3-aminophenyl-sulfonamido)-3-(3-cyanophenyl)propanoyl)piperidin-4-yl)ethylcarbamate

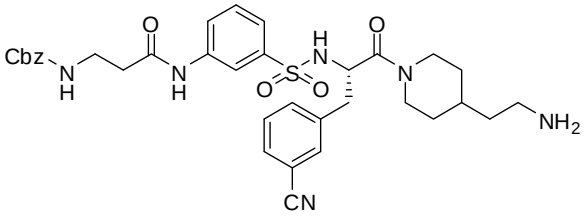
[Cbz-βAla-3-NH-phenylsulfonyl-Phe(3-CN)-4-Pip-Boc-ethylamine][78]



300 mg (0.539 mmol) intermediate **72** were coupled with 109.5 mg (0.491 mmol) Cbz-βAla-OH according to method L.
Yield: 300 mg, yellow oil.
HPLC: 63.7% B (purity: 95.33%).

74) (*S*)-benzyl 3-(3-(*N*-(1-(4-(2-aminoethyl)piperidin-1-yl)-3-(3-cyanophenyl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate × TFA

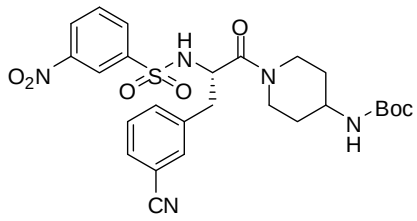
[Cbz-βAla-3-NH-phenylsulfonyl-l-Phe(3-CN)-4-Pip-ethylamine × TFA]



300 mg of intermediate **73** were deprotected with TFA according to method B.
Yield: 250 mg, yellow solid.
HPLC: 43.2% B (purity: 95.8%); MS calcd: 660.25, found: 661.28 $(M+H)^+$.

75) (*S*)-*tert*-butyl 1-(3-(3-cyanophenyl)-2-(3-nitrophenylsulfonamido)propanoyl)-piperidin-4-ylcarbamate

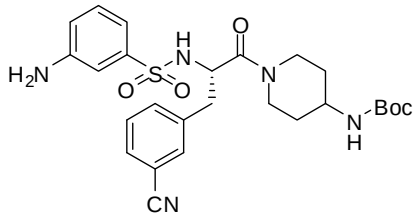
[3-NO_2-phenylsulfonyl-Phe(3-CN)-4-Pip-NH-Boc]



600 mg (1.59 mmol) intermediate **70** were coupled with 416.1 mg (1.76 mmol) *tert*-butyl piperidin-4-ylcarbamate × HCl according to method I.
Yield: 800 mg, orange solid.
HPLC: 59.2% B (purity: 93.9%).

76) (*S*)-*tert*-butyl 1-(2-(3-aminophenylsulfonamido)-3-(3-cyanophenyl)-propanoyl)piperidin-4-ylcarbamate

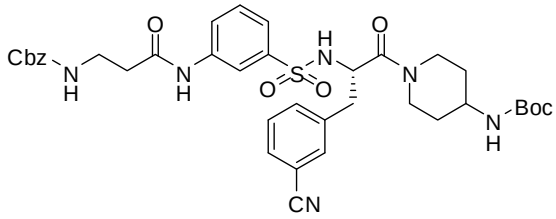
[3-NH_2-phenylsulfonyl-Phe(3-CN)-4-Pip-NH-Boc]



800 mg (≈ 1.434 mmol) intermediate **75** was reduced with zinc dust in 90% HOAc according to method Q.
Yield: 660 mg, orange oil.
HPLC: 49.02% B (purity: 83.8%), MS calcd: 527.22, found: 528.21 $(M+H)^+$.

77) Benzyl 3-amino-3-oxopropylcarbamate-(S)-*tert*-butyl 1-(2-(3-aminophenyl-sulfonamido)-3-(3-cyanophenyl)propanoyl)piperidin-4-ylcarbamate

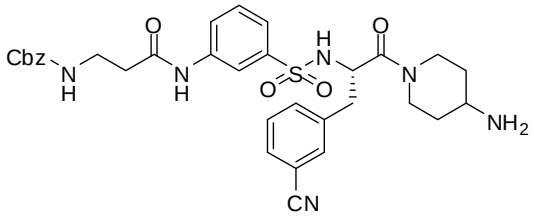
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-CN)-4-Pip-NH-Boc]



660 mg intermediate **76** were coupled with 254 mg (1.14 mmol) Cbz-βAla-OH according to method L.
Yield: 700 mg, yellow oil.
HPLC: 61.2% B (purity: 85.7%).

78) (*S*)-benzyl 3-(3-(*N*-(1-(4-aminopiperidin-1-yl)-3-(3-cyanophenyl)-1-oxo-propan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate × TFA

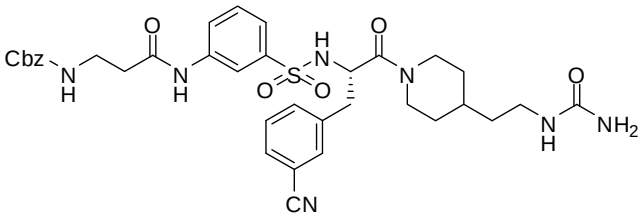
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-CN)-4-Pip-NH_2 × TFA]



700 mg intermediate **77** were deprotected with TFA according to method B.
Yield: 600 mg (≈ 0.803 mmol), yellow solid.
HPLC: 42.4% B (purity: 96.9%); MS calcd: 632.24, found: 633.31 $(M+H)^+$.

79) (*S*)-benzyl 3-(3-(*N*-(3-(3-cyanophenyl)-1-oxo-1-(4-(2-ureidoethyl)piperidin-1-yl)propan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

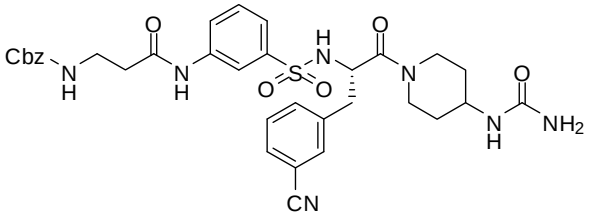
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-CN)-2-(piperidin-4-yl)Et-urea] [191]



50 mg (0.065 mmol) intermediate **74** were dissolved in 3 mL DCM, treated with 10 mg (0.097 mmol) *N*-methyl-*N*-nitrosourea and 18 μl (0.13 mmol) TEA. The mixture was stirred at RT overnight and the solvent was removed *in vacuo*. The remaining residue was dissolved in EtOAc, washed trice with saturated $NaHCO_3$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*.
Yield: 40 mg, oil.
HPLC: 50.8% B (purity: 71.3%).

80) (*S*)-benzyl 3-(3-(*N*-(3-(3-cyanophenyl)-1-oxo-1-(4-ureidopiperidin-1-yl)-propan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

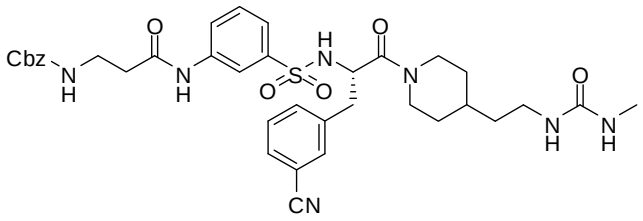
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-CN)-4-Pip-urea][191]



The compound **80** was prepared in analogy to the derivative **79** from treating 50 mg (0.067 mmol) intermediate **78** with 10.4 mg (0.100 mmol) *N*-methyl-*N*-nitrosourea and 18.7 μl (0.134 mmol) TEA in DCM.
Yield: 30 mg, oil.
HPLC: 48.2% B (purity: 80.7%).

81) (*S*)-benzyl 3-(3-(*N*-(3-(3-cyanophenyl)-1-(4-(2-(3-methylureido)ethyl)-piperidin-1-yl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

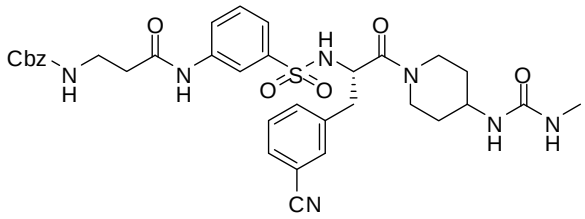
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-CN)-4-Pip-Et-methylurea][192]



50 mg intermediate **74** were dissolved in 3 mL DCM, treated with 16.7 mg (0.097 mmol) *N*-succinimidyl-*N*-methylcarbamate[190, 191] and 18 μl (0.13 mmol) TEA. The mixture was stirred at RT overnight and the solvent was removed *in vacuo*. The remaining residue was dissolved in EtOAc, washed trice with saturated $NaHCO_3$-solution and trice with brine. The EtOAc was dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.
Yield: 33 mg, oil.
HPLC: 51.2% B (purity: 89.9%).

82) (*S*)-benzyl 3-(3-(*N*-(3-(3-cyanophenyl)-1-(4-(3-methylureido)piperidin-1-yl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

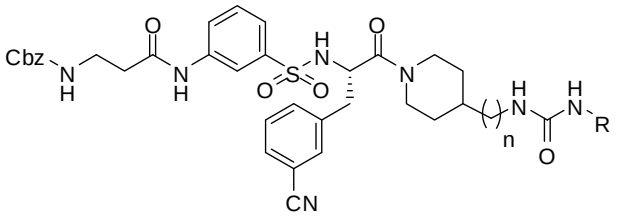
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-CN)-4-Pip-methylurea][192]

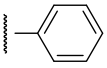
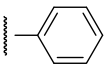
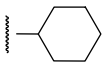
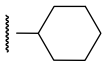
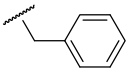
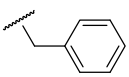


The compound **82** was prepared in analogy to the derivative **81** from treating 50 mg intermediate **78** with 17.3mg (0.101 mmol) *N*-succinimidyl-*N*-methylcarbamate and 18.7 μl (0.134 mmol) TEA in DCM.
Yield: 29 mg, yellowish solid.
HPLC: 50.3% B (purity: 83.9%).

Additional analogues **83-90** (Table 5.9) were analogous prepared as described for compound **81** and **82** according to method R using 50 mg intermediate **74** or 50 mg intermediate **78** and various isocyanate derivatives.

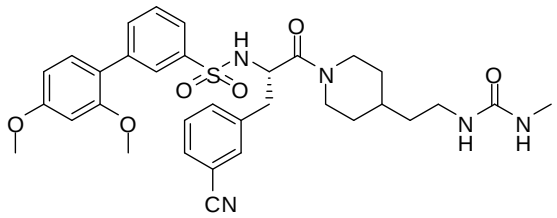
Table 5.9: Synthesis, yield, and analytical characterization of compounds **83-90** having the general formula.



No.	n	R	used isocyanates mg/mmol	Yield mg	HPLC % B, purity %
83	2	Et	ethylisocyanate 6.9/0.097	25	53.3, 90.3
84	0	Et	ethylisocyanate 7.2/0.101	30	52.4, 79.8
85	2		phenyl-isocyanate 11.6/0.097	40	60.3, 91.6
86	0		phenyl-isocyanate 12/0.101	35	58.8, 85.5
87	2		cyclohexyl-isocyanate 12.1/0.097	43	62.5, 95.5
88	0		cyclohexyl-isocyanate 12.6/0.101	38	60.4, 79.9
89	2		benzyl-isocyanate 13/0.097	35	60.2, 86.6
90	0		benzyl-isocyanate 13.4/0.101	33	58.8, 80.9

91) (*S*)-*N*-(3-(3-cyanophenyl)-1-(4-(2-(3-methylureido)ethyl)piperidin-1-yl)-1-oxopropan-2-yl)-2',4'-dimethoxybiphenyl-3-sulfonamide

[2′,4′-dimethoxbiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-Et-methylurea][192]



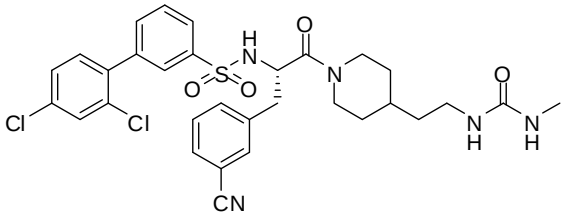
40 mg (≈ 0.069 mmol) intermediate **68** were treated with 18 mg (0.104 mmol) *N*-succinimidyl-*N*-methylcarbamate according to the procedure used for the synthesis of compound **81**.

Yield: 41 mg, yellowish solid.

HPLC: 54.4% B (purity: 93.4%).

92) (*S*)-2',4'-dichloro-*N*-(3-(3-cyanophenyl)-1-(4-(2-(3-methylureido)ethyl)-piperidin-1-yl)-1-oxopropan-2-yl)biphenyl-3-sulfonamide

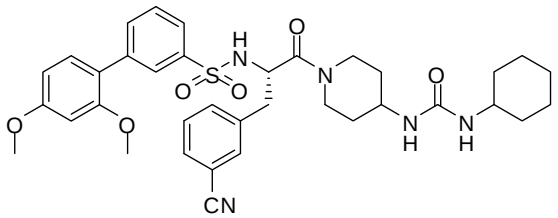
[2′,4′-dichlorobiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-Et-methylurea][192]



40 mg (≈ 0.068 mmol) intermediate **69** were treated with 18 mg (0.102 mmol) *N*-succinimidyl-*N*-methylcarbamate as described for the synthesis of intermediate **81**.
Yield: 40 mg, yellowish solid.
HPLC: 60.7% B (purity: 87.7%).

93) (*S*)-*N*-(3-(3-cyanophenyl)-1-(4-(3-cyclohexylureido)piperidin-1-yl)-1-oxo-propan-2-yl)-2',4'-dimethoxybiphenyl-3-sulfonamide

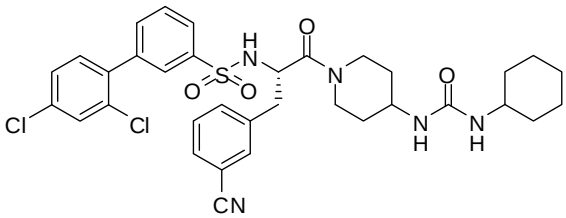
[3-(2′,4′-dimethoxybiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-Chxu]



38 mg (≈ 0.069 mmol) intermediate **65** were treated with 13 µl (0.104 mmol) cyclohexyl isocyanate according to method R.
Yield: 40 mg, yellow oil.
HPLC: 63.9.5% B (purity: 59.9%).

94) (*S*)-2',4'-dichloro-*N*-(3-(3-cyanophenyl)-1-(4-(3-cyclohexylureido)piperidin-1-yl)-1-oxopropan-2-yl)biphenyl-3-sulfonamide

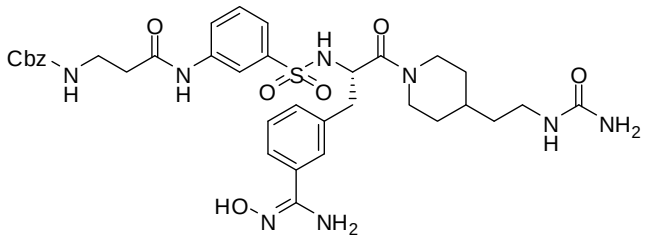
[3-(2′,4′-dichlorobiphenyl-3-sulfonyl-Phe(3-CN)-4-Pip-Chxu]



34 mg (≈ 0.061 mmol) intermediate **67** were treated with 12 µl (0.092 mmol) cyclohexylisocyanate according to method R.
Yield: 36 mg, yellow oil.
HPLC: 70.5% B (purity: 76.3%).

95) (*S*)-benzyl 3-(3-(*N*-(3-(3-(*N*'-hydroxycarbamimidoyl)phenyl)-1-oxo-1-(4-(2-ureidoethyl)piperidin-1-yl)propan-2-yl)sulfamoyl)phenylamino)-3-oxopropyl-carbamate

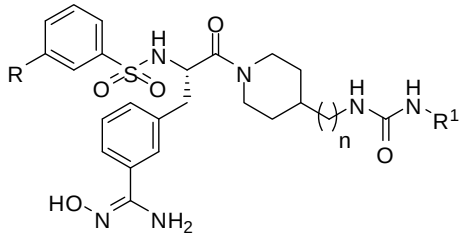
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-OxAm)-4-Pip-Et-urea]

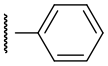
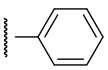
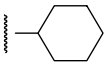
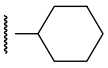
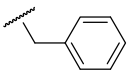
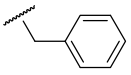
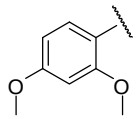
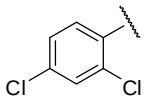
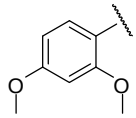
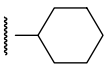
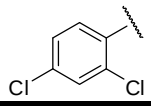
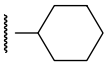


40 mg of the nitrile intermediate **79** were converted into hydroxyamidine using method O. The solvent was removed *in vacuo* and the remaining residue was used for the synthesis of intermediate **111** without further purification.
Yield: not determined, yellow oil.
HPLC: 38.7% B (purity: 85.7%).

Additional analogues **96-110** (Table 5.10) were prepared by treating the intermediates **80-94** according to method O.

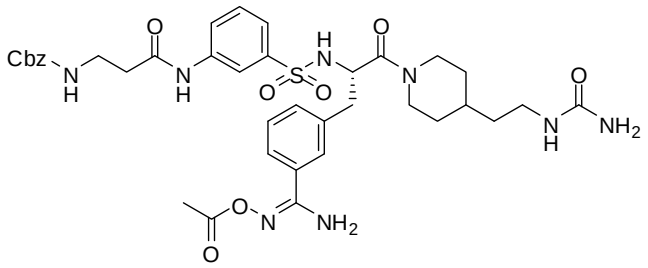
Table 5.10: Synthesis, yield and analytical characterization of compounds **96-110** having the general formula.



No.	n	R	R^1	Amount Educt		HPLC
				Intermediate No.	mg	% B, purity %
96	0	Cbz-*β*Ala-NH	H	**80**	30	37.4, 89.6
97	2	Cbz-*β*Ala-NH	Me	**81**	33	41.1, 80.2
98	0	Cbz-*β*Ala-NH	Me	**82**	29	38.3, 91.7
99	2	Cbz-*β*Ala-NH	Et	**83**	25	41.6, 89
100	0	Cbz-*β*Ala-NH	Et	**84**	30	40.2, 89.9
101	2	Cbz-*β*Ala-NH		**85**	40	47.7, 90.7
102	0	Cbz-*β*Ala-NH		**86**	35	46.5, 70.4
103	2	Cbz-*β*Ala-NH		**87**	43	48.9, 85.3
104	0	Cbz-*β*Ala-NH		**88**	38	47.2, 80.7
105	2	Cbz-*β*Ala-NH		**89**	35	47.3, 69.7
106	0	Cbz-*β*Ala-NH		**90**	33	46.5, 71.4
107	2		Me	**91**	41	41.8, 90.1
108	2		Me	**92**	40	46.6, 91.5
109	0			**93**	40	48.7, 85.6
110	0			**94**	36	53.6, 89.2

111) (*S*)-benzyl 3-(3-(*N*-(3-(3-(*N*'-acetoxycarbamimidoyl)phenyl)-1-oxo-1-(4-(2-ureidoethyl)piperidin-1-yl)propan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

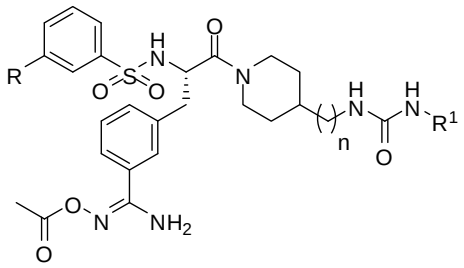
[Cbz-βAla-3-NH-phenylsulfonyl-Phe(3-AcOxAm)-4-Pip-Et-urea]

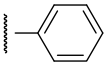
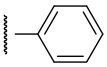
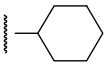
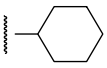
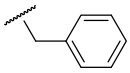
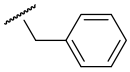
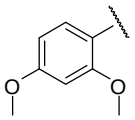
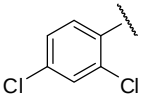
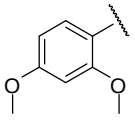
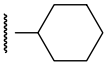
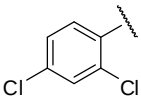
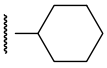


The intermediate **95** was acetylated according to method P. The remaining residue was used for the synthesis of compound **227** without further purification.
Yield: not determined, dark yellow oil with salts.
HPLC: 45.9% B (purity: 69%).

Additional analogues **112-126** (Table 5.11) were prepared by treating the intermediates **96-110** according to method P.

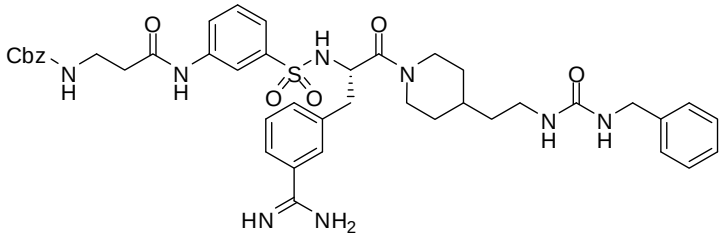
Table 5.11: Synthesis, yield and analytical characterization of compounds **112-126** having the general formula.



No.	n	R	R^1	Educt Intermediate No.	HPLC % B, purity %
112	0	Cbz-*β*Ala-NH	H	**96**	44.9, 53.1
113	2	Cbz-*β*Ala-NH	Me	**97**	49.3, 67.6
114	0	Cbz-*β*Ala-NH	Me	**98**	47.2, 52.9
115	2	Cbz-*β*Ala-NH	Et	**99**	50.1, 70.9
116	0	Cbz-*β*Ala-NH	Et	**100**	49.1, 74.4
117	2	Cbz-*β*Ala-NH		**101**	57.8, 77.1
118	0	Cbz-*β*Ala-NH		**102**	56.6, 55.6
119	2	Cbz-*β*Ala-NH		**103**	57.6, 69.5
120	0	Cbz-*β*Ala-NH		**104**	57.0, 53.5
121	2	Cbz-*β*Ala-NH		**105**	56.3, 67.7
122	0	Cbz-*β*Ala-NH		**106**	55.3, 45.6
123	2		Me	**107**	48.9, 59.5
124	2		Me	**108**	55.6, 55.2
125	0			**109**	60.4, 56.6
126	0			**110**	66.4, 39.3

127) (*S*)-benzyl 3-(3-(*N*-(1-(4-(2-(3-benzylureido)ethyl)piperidin-1-yl)-3-(3-carbamimidoylphenyl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

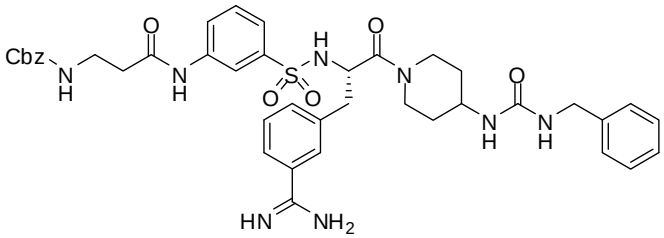
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-Am)-4-Pip-Et-Bzlu]



The intermediate **121** was reduced by zinc dust in 90% HOAc according to method Q. Compound **127** was used for the synthesis of inhibitor **MI_0446** without further purification.
Yield: not determined, light brown oil.
HPLC: 47.3% B (purity: 75%).

128) (*S*)-benzyl 3-(3-(*N*-(1-(4-(3-benzylureido)piperidin-1-yl)-3-(3-carbamimidoyl-phenyl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

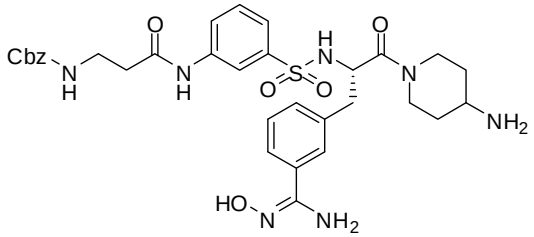
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-Am)-4-Pip-Bzlu]



The intermediate **122** was reduced by zinc dust in 90% HOAc according to method Q. Compound **128** was used for the synthesis of inhibitor **MI_0446** without further purification.
Yield: not determined, light brown oil.
HPLC: 46.1% B (purity: 82%).

129) (*S*)-benzyl 3-(3-(*N*-(1-(4-aminopiperidin-1-yl)-3-(3-(*N′*-hydroxycarbamimidoyl)phenyl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

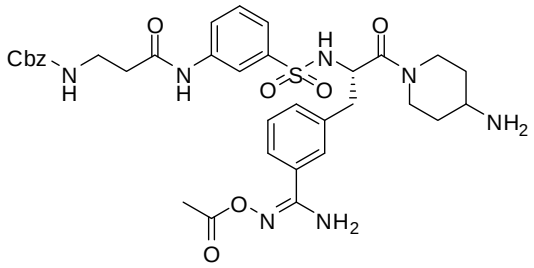
[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-OxAm)-4-Pip-NH_2]



50 mg (0.067 mmol) intermediate **78** were converted into hydroxyamidine using method O. The solvent was removed *in vacuo* and the remaining residue was used for the synthesis of intermediate **130** without further purification.
Yield: not determined, yellowish oil.
HPLC: 32.7% B (purity: 95.7%).

130) (*S*)-benzyl 3-(3-(*N*-(3-(3-(*N′*-acetoxycarbamimidoyl)phenyl)-1-(4-aminopiperidin-1-yl)-1-oxopropan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

[Cbz-*β*Ala-3-NH-phenylsulfonyl-Phe(3-OxAm)-4-Pip-NH_2]

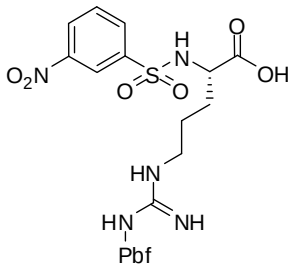


The intermediate **129** was acetylated according to method P. The remaining residue was used for the synthesis of inhibitor **MI-0458** without further purification.
Yield: not determined, yellow oil.
HPLC: 39.845.9% B (purity: 70.2%).

5.4.2 Synthesis of arginine derivatives

131) (*S*)-2-(3-nitrophenylsulfonamido)-5-(3-(3,3,4,6,7-pentamethyl-1,3-dihydroisobenzofuran-5-ylsulfonyl)guanidino)pentanoic acid

[3-NO_2-phenylsulfonyl-Arg(Pbf)-OH]



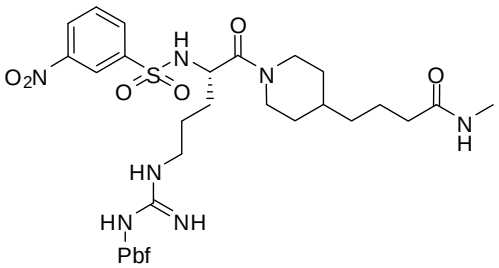
750 mg (1.758 mmol) H-Arg(Pbf)-OH were dissolved in a mixture of dioxane and water and treated with 428.5 mg (1.934 mmol) 3-nitrophenylsulfonyl chloride according to method M.

Yield: 1 g (1.635 mmol; 92.9%), yellowish solid.

HPLC: 55.7% B; MS calcd: 611.17, found: 611.97 $(M+H)^+$.

132) (*S*)-*N*-methyl-4-(1-(2-(3-nitrophenylsulfonamido)-5-(3-(3,3,4,6,7-pentamethyl-1,3-dihydroisobenzofuran-5-ylsulfonyl)guanidino)pentanoyl)piperidin-4-yl)butanamide

[3-NO_2-phenylsulfonyl-Arg(Pbf)-4-Pip-*N*-methylbutanamide]



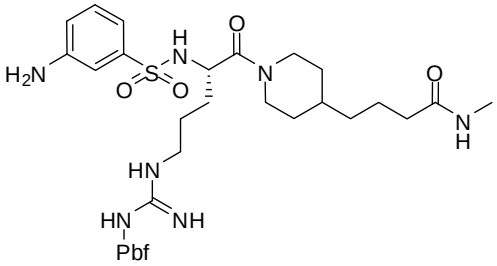
300 mg (0.49 mmol) intermediate **131** were coupled with 118.9 mg (0.539 mmol) intermediate **4** according to method I.

Yield: 327 mg, yellow oil.

HPLC: 56.4% B (purity: 87%).

133) (*S*)-4-(1-(2-(3-aminophenylsulfonamido)-5-(3-(3,3,4,6,7-pentamethyl-1,3-dihydroisobenzofuran-5-ylsulfonyl)guanidino)pentanoyl)piperidin-4-yl)-*N*-methylbutanamide

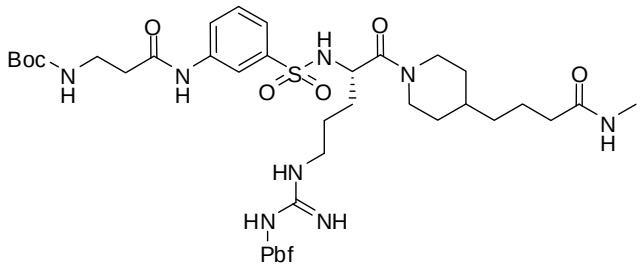
[3-NH_2-phenylsulfonyl-Arg(Pbf)-4-Pip-*N*-methylbutanamide]



208 mg intermediate **132** was reduced by zinc dust in 90% HOAc according to method Q.
Yield: 168 mg (≈ 0.225 mmol), yellowish crystals.
HPLC: 47.9% B (purity: 95%).

134) (*S*)-*tert*-butyl 3-(3-(*N*-(1-(4-(4-(methylamino)-4-oxobutyl)piperidin-1-yl)-1-oxo-5-(3-(3,3,4,6,7-pentamethyl-1,3-dihydroisobenzofuran-5-ylsulfonyl)-guanidino)pentan-2-yl)sulfamoyl)phenylamino)-3-oxopropylcarbamate

[Boc-*β*Ala-3-NH-phenylsulfonyl-Arg(Pbf)-4-Pip-*N*-methylbutanamide]

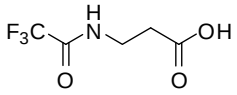


125 mg (0.167 mmol) intermediate **133** were coupled with 31.6 mg (0.167 mmol) Boc-*β*-alanine according to method L.
Yield: 125 mg, brown oil.
HPLC: 58.2% B (purity: 91%).

5.4.3 Synthesis of propargylglycine derivatives their precursors

135) 3-(2,2,2-trifluoroacetamido)propanoic acid

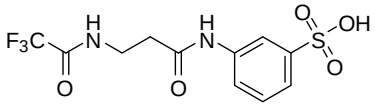
[Tfa-*β*Ala-OH][238]



9.5 g (0.106 mmol) *β*-alanine was acylated according to method G.
Yield: 17.7 g (0.097 mmol; 91.7%), white powder.
TLC (4:1:1) R_f: 0.42, MS calcd: 185.03, MS found: 184.05 $(M-H)^-$.

136) 3-(3-(2,2,2-trifluoroacetamido)propanamido)benzenesulfonic acid

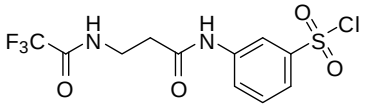
[Tfa-*β*Ala-NH-phenylsulfonic acid]



1 g (5.402 mmol) intermediate **135** was coupled with 1.03 g (5.942 mmol) metanilic acid (dissolved in DMF in presence of 1 equiv NMM) according to method L.
Yield: 1.6 g (4.702 mmol 87%), yellow powder.
HPLC 2: 27.3% B (purity: 92%); MS calcd: 340.03, found: 339.04 $(M-H)^-$.

137) 3-(3-(2,2,2-trifluoroacetamido)propanamido)benzene-1-sulfonyl chloride

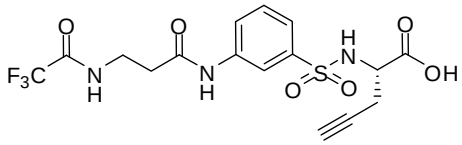
[Tfa-*β*Ala-3-NH-phenylsulfonylchloride][78]



500 mg (1.427 mmol) intermediate **136** were dissolved in 10 mL dry acetone and treated with 227 μl (1.62 mmol) TEA, 325 mg (1.764 mmol) cyanuric chloride and 21 mg (0.081 mmol) 18-crown-6. The mixture was stirred and heated under reflux for 20 h. The solvent was removed and the residue was dissolved in DCM and extracted with water. The organic phase was dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.
Yield: 500 mg, brown oil.

138) (*S*)-2-(3-(3-(2,2,2-trifluoroacetamido)propanamido)phenylsulfonamido)pent-4-ynoic acid

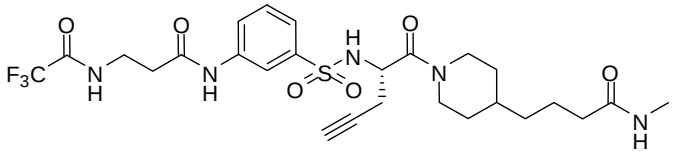
[Tfa-*β*Ala-3-NH-phenylsulfonyl-Pra-OH]



208.5 mg (1.39 mmol) H-L-Pra-OH × HCl were dissolved in a mixture of ACN and water in presence of 483 µl (2.78 mmol) DIPEA (pH ≈ 8.5-9). This mixture was treated with 500 mg (1.39 mmol) intermediate **137** according to method M and the pH was maintained at 8.5-9 with DIPEA.
Yield: 525 mg (≈ 1.205 mmol), brown solid.
HPLC 2: 33.13% B (purity: 75%); MS calcd: 435.37, found: 434.08 $(M-H)^-$.

139) (*S*)-*N*-methyl-4-(1-(2-(3-(3-(2,2,2-trifluoroacetamido)propanamido)-phenylsulfonamido)pent-4-ynoyl)piperidin-4-yl)butanamide

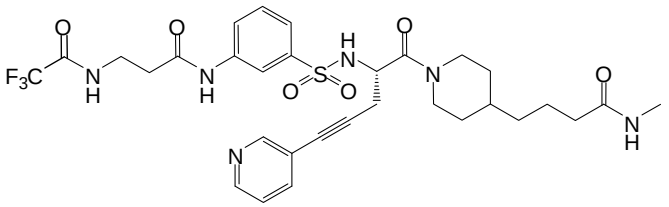
[Tfa-*β*Ala-3-NH-phenylsulfonyl-Pra-4-Pip-*N*-methylbutanamide]



500 mg (1.15 mmol) intermediate **138** were coupled with 279.2 mg (1.265 mmol) intermediate **4** according to method I.
Yield: 500 mg, brown oil.
HPLC 2: 39.3% B (purity: 75%), MS calcd: 601.22, found: 602.11 $(M+H)^+$.

140) (*S*)-*N*-methyl-4-(1-(5-(pyridin-3-yl)-2-(3-(3-(2,2,2-trifluoroacetamido)-propanamido)phenylsulfonamido)pent-4-ynoyl)piperidin-4-yl)butanamide

[Tfa-*β*Ala-3-NH-phenylsulfonyl-Pra-5-(pyridin-3-yl)-4-Pip-*N*-methylbutanamide]

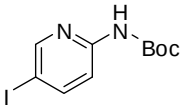


0.33 mg (0.001 mmol, 0.025 equiv) CuI, 46.5 µl (0.332 mmol, 8 equiv) TEA, 8.5 mg (0.041 mmol) 3-iodopyridine and 1.2 mg (0.001 mmol, 0.025 equiv) tetrakis(triphenylphosphine)palladium (0) were suspended in 3 mL ACN under argon. The stirred mixture was heated under reflux. After 1 h, 25 mg (0.041 mmol) of intermediate **139** dissolved in 1.5 mL ACN were added. The mixture was refluxed under argon for additional 18 h. After centrifugation, the supernatant was evaporated. The remaining residue was dissolved in EtOAc and washed trice with brine. The EtOAc was dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.

Yield: 20 mg, brown oil.
HPLC: 36.8% B (purity: 55%), MS calcd: 678.24, found: 679.20 $(M+H)^+$.

141) *tert*-butyl 5-iodopyridin-2-ylcarbamate

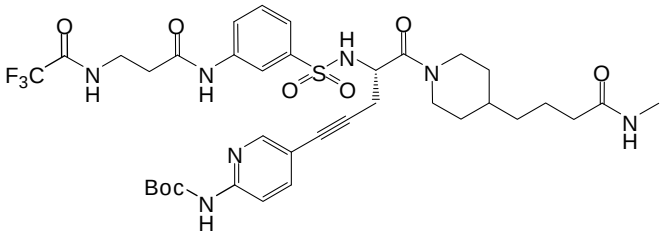
[2-(Boc-amino)-5-iodopyridine][246]



500 mg (2.27 mmol) 5-iodopyridin-2-amine were slowly added to a solution of 545.6 mg (2.49 mmol) $(Boc)_2O$ in distilled *tert*-butanol (0.169 mmol/mL) and the mixture was stirred at 25 °C for 24 h. The solvent was evaporated and the remaining residue was dissolved in EtOAc, washed trice with 5% $KHSO_4$-solution and trice with brine. The EtOAc phase was dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.
Yield: 710 mg (2.22 mmol; 98%), yellowish crystals.
HPLC: 48.3% B, TLC (4:1:1) R_f 0.9 MS calcd: 320.00, found: 320.98 $(M+H)^+$.

142) (*S*)-*tert*-butyl 5-(5-(4-(4-(methylamino)-4-oxobutyl)piperidin-1-yl)-5-oxo-4-(3-(3-(2,2,2-trifluoroacetamido)propanamido)phenylsulfonamido)pent-1-ynyl)-pyridin-2-ylcarbamate

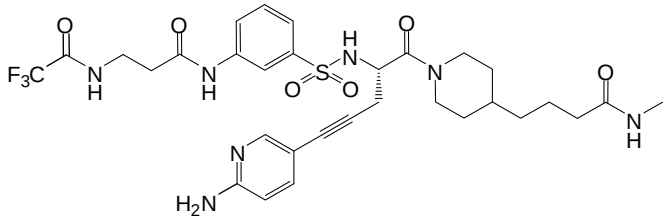
[Tfa-*β*Ala-3-NH-phenylsulfonyl-Pra-5-(6-Boc-aminopyridin-3-yl)-4-Pip-*N*-methylbutanamide]



25 mg intermediate **139** were coupled with 13.3 mg (0.041 mmol) compound **141** according to the Sonogashira protocol described for the synthesis of analogue **140.**
Yield: 21 mg, brown oil.
HPLC: 53.7% B (purity: 64%).

143) (*S*)-4-(1-(5-(6-aminopyridin-3-yl)-2-(3-(3-(2,2,2-trifluoroacetamido)propanamido)phenylsulfonamido)pent-4-ynoyl)piperidin-4-yl)-*N*-methylbutanamide

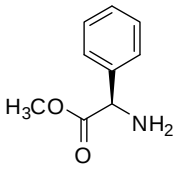
[Tfa-*β*Ala-3-NH-phenylsulfonyl-Pra-5-(6-aminopyridin-3-yl)-4-Pip-*N*-methylbutanamide]



The crude intermediate **142** was deprotected in a mixture of DCM and TFA (1:1, v/v) for 1 h. The solvent was evaporated and the remaining residue was used for the synthesis of inhibitor **MI-0408** without further purification.
Yield: not determined, brown oil.
HPLC: 36.7% B (purity: 85%), MS calcd: 693.26, found: 694.13 $(M+H)^+$.

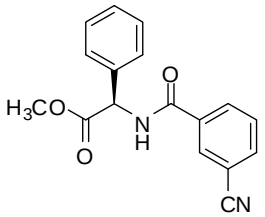
5.4.4 Synthesis of D-phenylglycine derivatives

144) (*R*)-methyl 2-amino-2-phenylacetate × HCl[204]



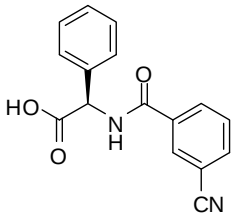
3.44 mL (47.31 mmol) thionyl chloride were added in portions to 13.5 mL (327 mmol) MeOH at -10 °C and stirred for 10 min. 2 g (13.23 mmol) D-phenylglycine were added to the thionyl chloride solution. The mixture was stirred at -10 °C for 30 min and at RT for additional 24 h. The solvent was concentrated *in vacuo* to a volume of 5 mL. After addition of 3 mL MeOH the product was crystallized at 4 °C. The product was filtered and dried *in vacuo*.
Yield: 2.5 g (12.39 mmol; 93.7%), white powder.
HPLC: 17.35% B.

145) (*R*)-methyl 2-(3-cyanobenzamido)-2-phenylacetate



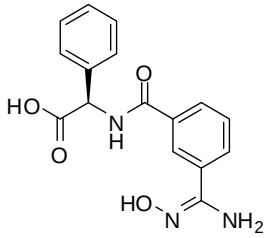
1.09 g (7.44 mmol) 3-cyanobenzoic acid was coupled with 1.5 g (7.44 mmol) intermediate **144** according to method L.
Yield: 1.8 g (6.12 mmol; 82.2%), white solid.
HPLC: 49.5% B.

146) (*R*)-2-(3-cyanobenzamido)-2-phenylacetic acid



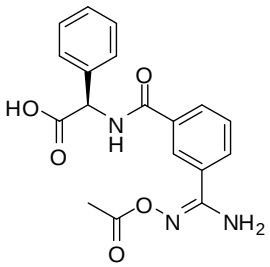
1.8 g (6.12 mmol) intermediate **145** were dissolved in a mixture of dioxane and water (1:1, 10 mL/mmol) and saponified using 10 mL 1 N NaOH. The pH was adjusted to 12 with 1 N NaOH and the mixture was stirred at RT for 3 h (HPLC control). After completion of the reaction, the mixture was acidified by 5% $KHSO_4$-solution and extracted trice with EtOAc. The organic phase was collected, washed trice with brine, dried over Na_2SO_4, filtered and the solvent was removed *in vacuo*.
Yield: 1.6 g (5.71 mmol; 93.2%), white solid.
HPLC: 41.7% B (purity: 98%).

147) (*R*)-2-(3-(*N*'-hydroxycarbamimidoyl)benzamido)-2-phenylacetic acid



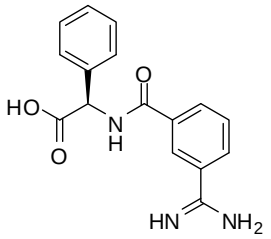
1 g (3.57 mmol) of the nitrile **146** was converted into hydroxyamidine according to method O. The remaining residue was used for the synthesis of intermediate **148** without further purification.
Yield: not determined, white powder.
HPLC: 24.8% B (purity: 94%).

148) (*R*)-2-(3-(*N*'-acetoxycarbamimidoyl)benzamido)-2-phenylacetic acid



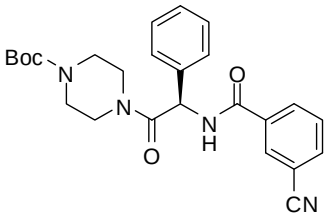
The crude intermediate **147** was acetylated according to method P. The residue was used for the synthesis of intermediate **149** without further purification.
Yield: not determined, white solid.
HPLC: 34.6% B (purity: 85%).

149) (*R*)-2-(3-carbamimidoylbenzamido)-2-phenylacetic acid × TFA



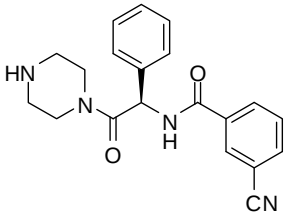
The crude intermediate **148** was hydrogenated according to method F and purified by preparative HPLC.
Yield: 820 g (1.99 mmol; 55.7%), white lyophilized solid.
HPLC: 24.8% B, MS calcd: 297.11, found: 298.31 $(M+H)^+$.

150) (*R*)-*tert*-butyl 4-(2-(3-cyanobenzamido)-2-phenylacetyl)piperazine-1-carboxylate



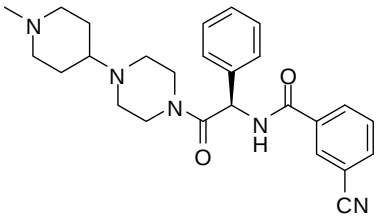
350 mg (1.25 mmol) intermediate **146** were coupled with 255.9 mg (1.373 mmol) Boc-piperazine according to method L.
Yield: 500 mg (1.115 mmol), yellow oil.
HPLC: 58.4% B (purity: 87%).

151) (*R*)-3-cyano-*N*-(2-oxo-1-phenyl-2-(piperazin-1-yl)ethyl)benzamide × HCl



500 mg intermediate **150** were deprotected according to method C.
Yield: 400 mg (1.039 mmol)**,** yellow oil.
HPLC: 30.9% B (purity: 75%).

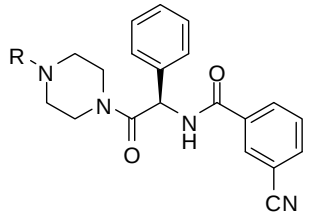
152) (*R*)-3-cyano-*N*-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)benzamide

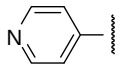
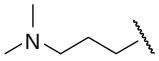
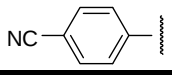


50 mg (0.178 mmol) intermediate **146** were coupled with 21.6 μl (0.178 mmol) 1-(1-methylpiperidin-4-yl)piperazine according to method L.
Yield: 35 mg, yellowish solid.
HPLC: 29.1% B (purity: 66.2%); MS calcd: 445.25, found: 446.20 $(M+H)^+$.

Additional analogues **153-155** (Table 5.12) were prepared by coupling 50 mg intermediate **146** with 0.178 mmol (1.0 equiv) of various piperazine derivatives according to method L.

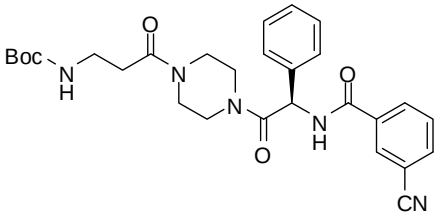
Table 5.12: Synthesis, yield and analytical characterization of compounds **153-155** having the general formula.



No.	R	used piperazine mg	Yield[a] mg	HPLC % B, purity %	MS calcd, found $(M+H)^+$
153		1-(pyridin-4-yl)piperazine 29.1	40	35.6,	425.19, 426.11
154		dimethyl-3-(piperazin-1-yl)propan-1-amine 30.5	50	29.3,	433.25, 434.01
155		4-piperazine-1-yl-benzonitrile 33.3	55	57.1,	449.19, 450.40

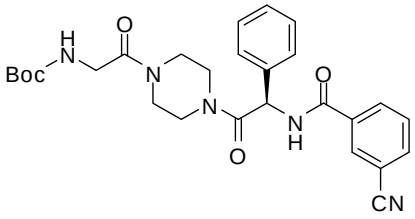
[a]yellow oil

156) (*R*)-*tert*-butyl 3-(4-(2-(3-cyanobenzamido)-2-phenylacetyl)piperazin-1-yl)-3-oxopropylcarbamate



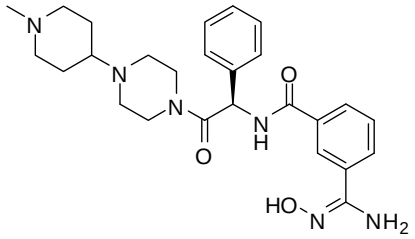
150 mg intermediate **151** were coupled with 73.7 mg (0.389 mmol) Boc-βAla-OH according to method J.
Yield: 165 mg, yellow oil.
HPLC: 51.2% B (purity: 58%); MS calcd: 519.25, found: 541.80 $(M+Na)^+$.

157) (*R*)-*tert*-butyl 2-(4-(2-(3-cyanobenzamido)-2-phenylacetyl)piperazin-1-yl)-2-oxoethylcarbamate



100 mg intermediate **151** were coupled with 45.5 mg (0.259 mmol) Boc-Gly-OH according to method L.
Yield: 80 mg, yellow oil.
HPLC: 50.2% B (purity: 70%).

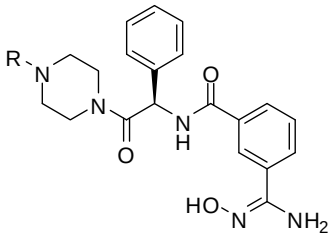
158) (*R*)-3-(*N*'-hydroxycarbamimidoyl)-*N*-(2-(4-(1-methylpiperidin-4-yl)-piperazin-1-yl)-2-oxo-1-phenylethyl)benzamide

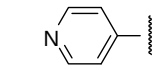
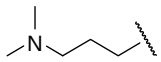
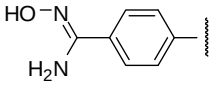
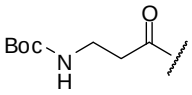
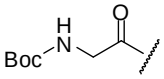


35 mg nitrile intermediate **152** were converted into the hydroxyamidine according to method O. The solvent was removed *in vacuo* and the residue was used for the synthesis of intermediate **164** without further purification.
Yield: not determined, yellow oil.
HPLC: 17.1% B (purity: 80.2%).

Additional hydroxyamidine derivatives **159-163** (Table 5.13) were prepared from intermediates **153-157** as described for compound **158**.

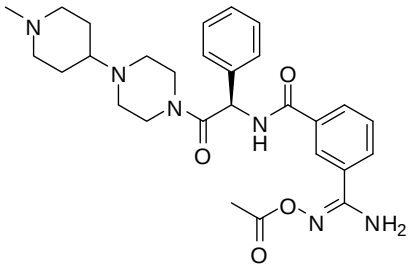
Table 5.13: Synthesis, yield and analytical characterization of compounds **159-163** having the general formula.



No.	R	Educt		HPLC
		Intermediate. No.	mg	% B, purity %
159		**153**	40	23.7, 89.4
160		**154**	50	18.6, 75
161*		**155**	55	27.3, 83.2
162		**156**	165	36.8, 80
163		**157**	80	34.8, 90

*6 equiv (50 mg, 0.732 mmol) $NH_2OH \times HCl$ and 6 equiv (127 μl, 0.732 mmol) DIPEA were used to convert both nitrile groups.

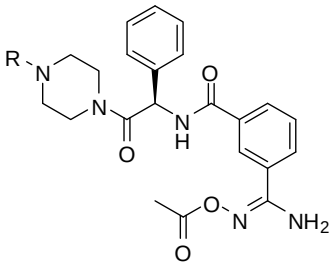
164) (*R*)-3-(*N*'-acetoxycarbamimidoyl)-*N*-(2-(4-(1-methylpiperidin-4-yl)-piperazin-1-yl)-2-oxo-1-phenylethyl)benzamide

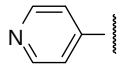
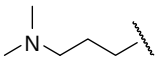
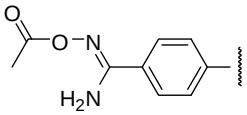
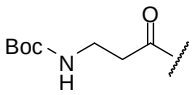
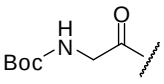


The crude intermediate **158** was acetylated according to method P. The remaining residue was used for the synthesis of inhibitor **MI-0416** without further purification.
Yield: not determined, yellow oil.
HPLC: 24.8% B (purity: 61%).

Additional analogues **165-169** (Table 5.14) were prepared by acetylation of the intermediates **159-163** as described for analogue **164**.

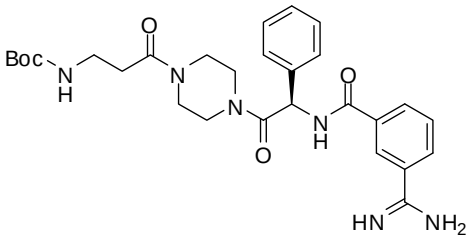
Table 5.14: Synthesis, yield and analytical characterization of compounds **165-169** having the general formula.



No.	R	Educt: Intermediate No.	HPLC % B, purity %
165		**159**	31.1, 65.3
166		**160**	27.1, 75.1
167*		**161**	40.9, 65.6
168		**162**	45.2, 78.3
169		**163**	42.1, 64.5

*6 equiv acetic anhydride were used for acetylation of both hydroxyamidine groups.

170) (*R*)-tert-butyl 3-(4-(2-(3-carbamimidoylbenzamido)-2-phenylacetyl)-piperazin-1-yl)-3-oxopropylcarbamate

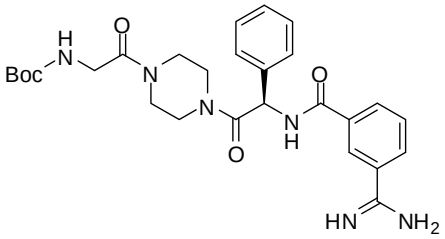


Intermediate **168** was hydrogenated according to method F. The solvent was removed *in vacuo* and the residue was used for the synthesis of inhibitor **MI-0429** without further purification.

Yield: not determined, yellow oil.

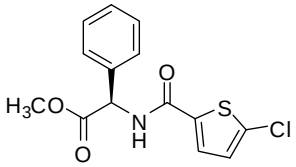
HPLC: 36.8% B (purity: 50%).

171) (*R*)-*tert*-butyl 2-(4-(2-(3-carbamimidoylbenzamido)-2-phenylacetyl)-piperazin-1-yl)-2-oxoethylcarbamate



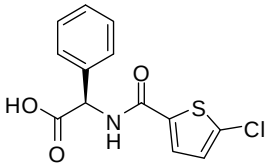
Compound **171** was prepared from the intermediate **169** as decribed for the analogue **170** according to method F.
Yield: not determined, yellow oil.
HPLC: 34.8% B (purity: 60%).

172) (*R*)-methyl 2-(5-chlorothiophene-2-carboxamido)-2-phenylacetate



150 mg (0.744 mmol) intermediate **144** were coupled with 120.9 mg (0.744 mmol) 5-chlorothiophene-2-carboxylic acid according to method L.
Yield: 195 mg (0.629 mmol; 84.6%), yellow solid.
HPLC: 57.3% B (purity: 80%); MS calcd: 309.02, found: 310.00 $(M+H)^+$.

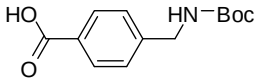
173) (*R*)-2-(5-chlorothiophene-2-carboxamido)-2-phenylacetic acid



300 mg intermediate **172** were saponified according to method H.
Yield: 250 mg, yellow crystallized product.
HPLC: 49.1% B (purity: 75%); MS calcd: 295.01, found: 296.01 $(M+H)^+$.

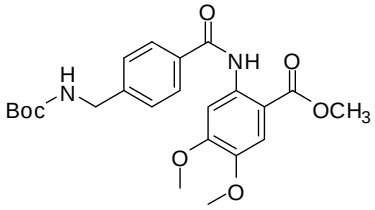
5.4.5 Synthesis of antranilamide derivatives and precursors

174) 4-((*tert*-butoxycarbonylamino)methyl)benzoic acid



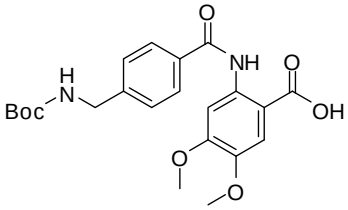
5 g (33.07 mmol) 4-(aminomethyl)benzoic acid were protected using $(Boc)_2O$ according to method A.
Yield: 8 g, yellow powder.
HPLC: 43.2% B (purity: 98%).

175) Methyl 2-(4-((*tert*-butoxycarbonylamino)methyl)benzamido)-4,5-dimethoxybenzoate



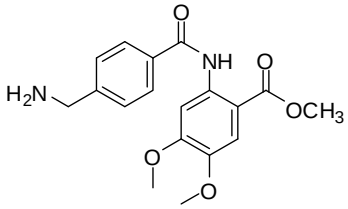
500 mg intermediate **174** were coupled with 462.3 mg (2.19 mmol) methyl 2-amino-4,5-dimethoxybenzoate according to method L.Yield: 510 g, oil.
HPLC: 65.9% B (purity: 80%); MS calcd: 444.19, found: 889.11 $(2M+H)^+$.

176) 2-(4-((*tert*-butoxycarbonylamino)methyl)benzamido)-4,5-dimethoxybenzoic acid



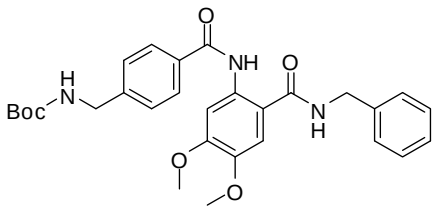
300 mg intermediate **175** were saponified according to method H.
Yield: 275 mg, white solid.
HPLC: 57.7% B (purity: 72%); MS calcd: 430.17, found: 431.01 $(M+H)^+$.

177) Methyl 2-(4-(aminomethyl)benzamido)-4,5-dimethoxybenzoate× HCl



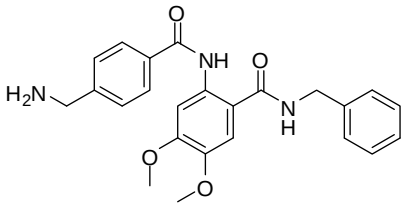
150 mg intermediate **175** were deprotected according to method C.Yield: 120 mg, white solid.
HPLC: 36.9% B (purity: 75%); MS calcd: 344.14, found: 345.01 $(M+H)^+$.

178) *tert*-butyl 4-(2-(benzylcarbamoyl)-4,5-dimethoxyphenylcarbamoyl)-benzylcarbamate



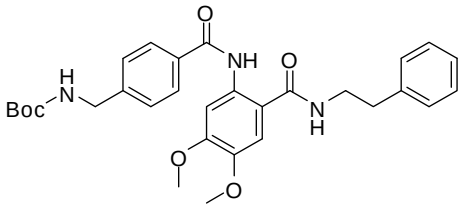
100 mg intermediate **176** were coupled with 27.85 μl (0.255 mmol) benzylamine according to method L.
Yield: 110 mg, yellow oil.
HPLC: 65.9% B (purity: 73%); MS calcd: 519.24, found: 520.36 $(M+H)^+$.

179) 2-(4-(Aminomethyl)benzamido)-*N*-benzyl-4,5-dimethoxybenzamide × HCl



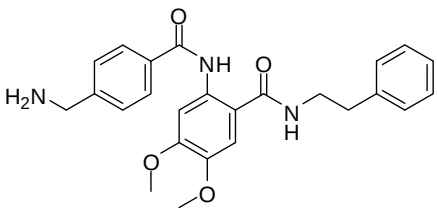
110 mg intermediate **178** were deprotected according to method C.
Yield: 90 mg, white solid.
HPLC: 42% B (purity: 99%); MS calcd: 419.18, found: 420.22 $(M+H)^+$.

180) *tert*-butyl 4-(4,5-dimethoxy-2-(phenethylcarbamoyl)phenylcarbamoyl)-benzylcarbamate



100 mg intermediate **176** were coupled with 32 μl (0.255 mmol) phenylethylamine according to method L.
Yield: 110 mg, yellow oil.
HPLC: 68.9% B (purity: 45%).

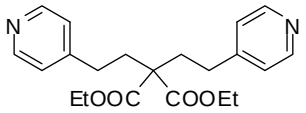
181) 2-(4-(Aminomethyl)benzamido)-4,5-dimethoxy-*N*-phenethylbenzamide



100 mg (0.187 mmol) intermediate **180** were deprotected according to method C.
Yield: 80 mg, white powder.
HPLC: 42.7% B (purity: 40%).

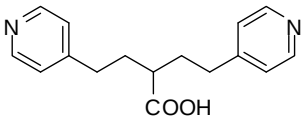
5.4.6 Synthesis of substrate analogue inhibitors and precursors

182) Diethyl 2,2-bis(2-(pyridin-4-yl)ethyl)malonate[228]



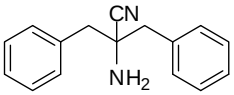
1 g (41.6 mmol) sodium hydride was dissolved in 75 mL absolute ethanol at 0 °C and treated with 3.75 mL (23.4 mmol) diethyl malonate. The mixture was heated to 80 °C and stirred for 30 min. The mixture was cooled to RT, treated with 2.71 g (25.74 mmol) 4-vinylpyridine and stirred under reflux for 3 h. The solvent was removed *in vacuo* and 1 g of the residue was purified by preparative HPLC.
Yield: 0.7 g (1.89 mmol), yellow oil.
HPLC: 21% B; MS calcd: 370.17, found: 371.07 $(M+H)^+$.

183) 4-(Pyridin-4-yl)-2-(2-(pyridin-4-yl)ethyl)butanoic acid × 2 TFA[229]



700 mg (1.89 mmol) intermediate **182** were dissolved in a mixture of 4 mL dioxane and water (1:1, v/v) and heated to 80 °C. 2 mL 3 N NaOH were added in several portions over a period of 3 h and the mixture was stirred at 95 °C for 4 h (HPLC control). An additional 2 mL 3 N NaOH were added and stirring was continued at 95 °C for additional 4 h. The residue was cooled to RT, neutralized with 1 N HCl and the solvent was evaporated. The product was purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 407 mg (0.82 mmol, 43.4%), white crystallized product.
HPLC: 12% B (purity: 100%); MS calcd: 270.14, found: 271.14 $(M+H)^+$.

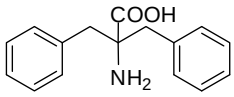
184) 2-Amino-2-benzyl-3-phenylpropanenitrile[231, 232, 247]



8.558 g (160 mmol) NH_4Cl and 7.105 g (145 mmol) NaCN were dissolved in 20 mL water and treated with 10 mL ammonia. To this mixture, 10.52 g (50 mmol) 1,3-diphenylpropan-2-one (Aldrich) dissolved in 10 mL MeOH were added in several portions. The mixture was stirred at 50 °C for 8 h and the reaction was controlled by HPLC. An additional 1.337 g (25 mmol) NH_4Cl, 1.225 g (25 mmol) NaCN and 3 mL ammonia were dissolved in 5 mL water and added to the reaction mixture. Stirring was continued at 50 °C for additional 24 h. The mixture was concentrated *in vacuo* to a small volume. The residue was acidified with 1 N HCl and extracted trice with EtOAc to remove the hydrophobic impurities. The water phase was used for the synthesis of intermediate **185**.
HPLC: 38.96% B; MS calcd: 236.13, found: 119.19 $(M+2H)^{2+}$.

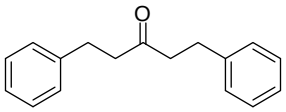
185) 2-Amino-2-benzyl-3-phenylpropanoic acid × TFA

[H-Dbzg-OH × TFA][231, 232, 247]



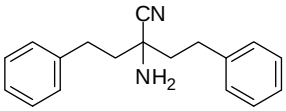
25 mL 32% HCl were added to the water phase of intermediate **184** and the mixture was stirred under refluxe for 48 h. The mixture was concentrated *in vacuo*, the residue was treated with water and evaporated. This procedure was repeated several times to remove HCl. 1 g of the residue was purified by preparative HPLC, and the product was lyophilized from 80% *tert*-butanol.
Yield: 1.3 g (3.52 mmol), yellow solid.
HPLC: 31.5% B; MS calcd: 255.13, found: 256.11 $(M+H)^+$.

186) 1,5-Diphenylpentan-3-one[248]



9.8 g (41.8 mmol) *trans*,*trans*-dibenzylideneacetone were dissolved in 500 mL EtOAc and treated with 100 mg catalyst (10%, m/m Pd/C). The mixture was hydrogenated at RT for 24 h. The catalyst was removed by filtration, the solvent was evaporated, and the product was purified by preparative HPLC.
Yield: 9.95 g (41.75 mmol 99.9%), colourless oil.
HPLC: 71.3% B; MS calcd: 238.14, found: 239.12 $(M+H)^+$.

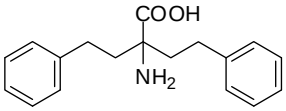
187) 2-Amino-2-phenethyl-4-phenylbutanenitrile[231, 232, 247]



2 g (8.39 mmol) of compound **186** were converted to intermediate **185** in analogy to the synthesis of derivative **184**.
HPLC: 45.8% B; MS calcd: 264.16, found: 265.18 $(M+H)^+$.

188) 2-amino-2-phenethyl-4-phenylbutanoic acid × TFA

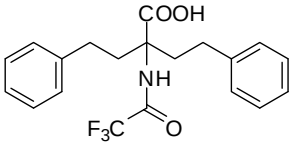
[H-Dpheg-OH × TFA]



Intermediate **187** was hydrolysed with HCl as described for the preparation of compound **185**.
1 g of the residue was purified by preparative HPLC and the product was lyophilized from 80% *tert*-butanol.
Yield: 470 mg (1.183 mmol), yellow solid.
HPLC: 41.1% B; MS calcd: 283.16, found: 284.13 $(M+H)^+$.

189) 2-phenethyl-4-phenyl-2-(2,2,2-trifluoroacetamido)butanoic acid

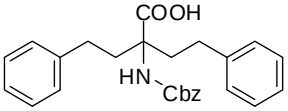
[Tfa-Dpheg-OH]



100 mg (0.251 mmol) intermediate **188** were protected using trifluroacetic anhydride according to method G.
Yield: 92 mg (0.242 mmol; 96.6%), yellowish oil.
HPLC: 67.7% B (purity: 100%).

190) 2-(benzyloxycarbonylamino)-2-phenethyl-4-phenylbutanoic acid

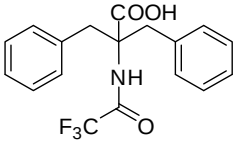
[Cbz-Dpheg-OH]



120 mg (0.317 mmole) intermediate **188** were protected using Cbz-OSu according to method D.
Yield: 125 mg (0.299 mmol; 94.4%), yellow solid.
HPLC: 70.5% B (purity: 99.8%).

191) 2-benzyl-3-phenyl-2-(2,2,2-trifluoroacetamido)propanoic acid

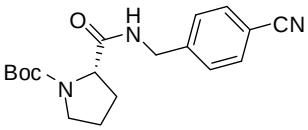
[Tfa-Dbzg-OH]



300 mg (0.812 mmol) intermediate **185** were protected using trifluroacetic anhydride according to method G.
Yield: 270 mg (0.769 mmol; 94.7%), yellow oil.
HPLC: 65.3% B (purity: 99.3%).

192) (S)-*tert*-butyl 2-(4-cyanobenzylcarbamoyl)pyrrolidine-1-carboxylate

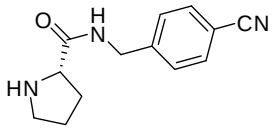
[Boc-Pro-4-cyanobenzylamide][249]



5 g (23.23 mmol) Boc-Pro-OH were coupled with 4.31 g (25.55 mmol) 4-cyanobenzylamine × HCl according to method L.
Yield: 7.11 g, oil.
HPLC: 47.1% B (purity: 99.2%).

193) (S)-*N*-(4-cyanobenzyl)pyrrolidine-2-carboxamide × HCl

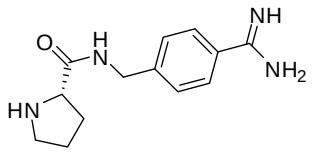
[H-Pro-4-cyanobenzylamide × HCl]



1.5 g (4.553 mmol) intermediate **192** were deprotected according to method C.
Yield: 1.15 g (4.328 mmol; 95.1%), beige solid.
HPLC: 22.3% B (purity: 100%).

194) (S)-*N*-(4-carbamimidoylbenzyl)pyrrolidine-2-carboxamide × 2 HCl

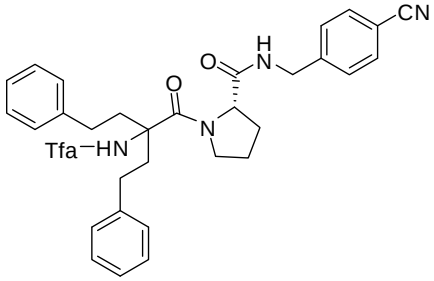
[H-Pro-(4-Am)benzylamide × 2 HCl][221, 249]



1 g (2.28 mmol) Cbz-Pro-(4-acetylhydroxyamidine)benzylamide (provided by Prof. Dr. Steinmetzer) were dissolved in 100 mL 90% HOAc and treated with 25 mg Pd/C. The mixture was stirred at RT for 48 h under hydrogen atmosphere. The catalyst was removed by filtration and the solvent was evaporated. The residue was dissolved in 1 N HCl and the solution was concentrated *in vacuo*. The remaining residue was dissolved again in water and evaporated.This procedure was repeated three times. The product was lyophilized from water.
Yield: 700 mg (2.19 mmol; 96.1%), beige solid.
HPLC: 11.8% B; TLC: R_f 0.10; MS calcd: 246.15, found: 247.14 $(M+H)^+$.

195) (S)-*N*-(4-cyanobenzyl)-1-(2-phenethyl-4-phenyl-2-(2,2,2-trifluoroacetamido)-butanoyl)pyrrolidine-2-carboxamide

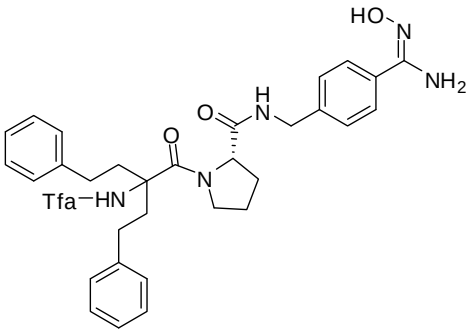
[Tfa-Dpheg-*N*-Pro-4-cyanobenzylamide]



85 mg (0.224 mmol) intermediate **189** were coupled with 65.5 mg (0.246 mmol) intermediate **193** according to method K.
Yield: 110 mg, brown oil.
HPLC: 71.5% B (purity: 35%); MS calcd: 590.25, found: 591.29 $(M+H)^+$.

196) (*S*)-*N*-(4-(*N′*-hydroxycarbamimidoyl)benzyl)-1-(2-phenethyl-4-phenyl-2-(2,2,2-trifluoroacetamido)butanoyl)pyrrolidine-2-carboxamide

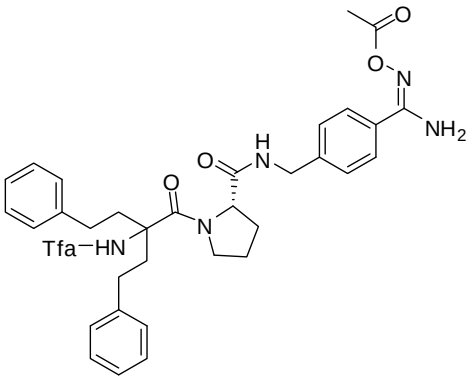
[Tfa-Dpheg-Pro-(4-OxAm)-benzylamide]



110 mg (0.186 mmol) of the nitrile **195** were converted into the hydroxyamidine according to method O and used for the synthesis of intermediate **197** without further purification.
Yield: not determined, brown oil.
HPLC: 53.07% B (purity: 55%).

197) (*S*)-*N*-(4-(*N′*-acetoxycarbamimidoyl)benzyl)-1-(2-phenethyl-4-phenyl-2-(2,2,2-trifluoroacetamido)butanoyl)pyrrolidine-2-carboxamide

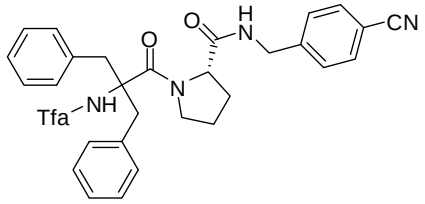
[Tfa-DPheg-Pro-(4-AcOxAm)-benzylamide]



0.186 mmol intermediate **196** was acetylated according to method P. The solvent was removed *in vacuo* (temperature of the water bath < 35 °C) and the residue was used for the synthesis of inhibitor **MI-0457** without further purification.
Yield: not determined, brown oil.
HPLC: 64.1% (purity: 45%).

198) (*S*)-1-(2-benzyl-3-phenyl-2-(2,2,2-trifluoroacetamido)propanoyl)-*N*-(4-cyanobenzyl)pyrrolidine-2-carboxamide

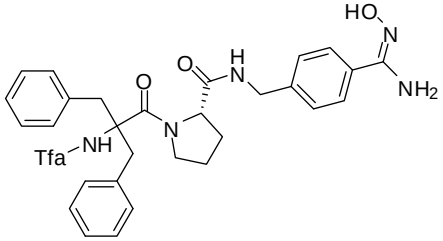
[Tfa-Dbzg-Pro-4-cyanobenzylamide]



250 mg (0.712 mmol) intermediate **191** were coupled with 208 mg (0.783 mmol) intermediate **193** according to method K.
Yield: 300 mg, brown oil.
HPLC: 67.7% B (purity: 59%); MS calcd: 562.22, found: 563.21 $(M+H)^+$.

199) (*S*)-1-(2-benzyl-3-phenyl-2-(2,2,2-trifluoroacetamido)propanoyl)-*N*-(4-(*N'*-hydroxycarbamimidoyl)benzyl)pyrrolidine-2-carboxamide

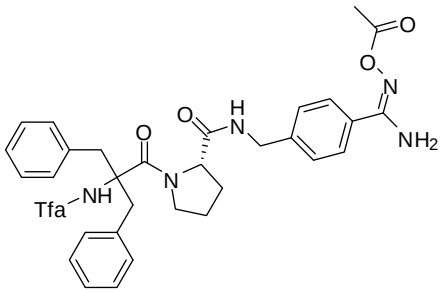
[Tfa-Dbzg-Pro-(4-OxAm)benzylamide]



300 mg (0.533 mmol) of the nitrile **198** were converted into hydroxyamidine according to method O and used for the synthesis of intermediate **200** without further purification.
Yield: not determined, brown oil.
HPLC: 48.3% B (purity: 59%).

200) (*S*)-*N*-(4-(*N'*-acetoxycarbamimidoyl)benzyl)-1-(2-benzyl-3-phenyl-2-(2,2,2-trifluoroacetamido)propanoyl)pyrrolidine-2-carboxamide

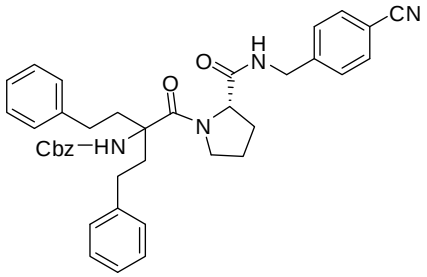
[Tfa-Dbzg-Pro-(4-AcOxAm)benzylamide]



Intermediate **199** were acetylated according to method P and used for the synthesis of inhibitor **MI-0456** without further purification.
Yield: not determined, brown oil.
HPLC: 59.5% B (purity: 52%).

201) (*S*)-benzyl 3-(2-(4-cyanobenzylcarbamoyl)pyrrolidine-1-carbonyl)-1,5-diphenylpentan-3-ylcarbamate

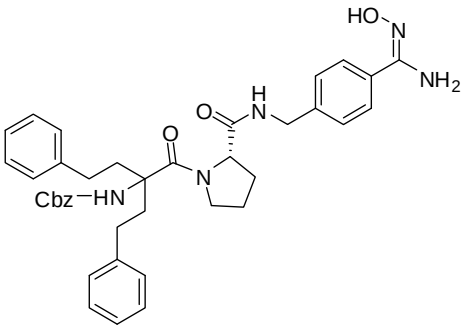
[Cbz-Dpheg-Pro-4-cyanobenzylamide]



100 mg (0.239 mmol) intermediate **190** were coupled with 70 mg (0.263 mmol) intermediate **193** according to method K.
Yield: 120 mg, brown oil.
HPLC: 72.5% B (purity: 40%).

202) (*S*)-benzyl 3-(2-(4-(N'-hydroxycarbamimidoyl)benzylcarbamoyl)-pyrrolidine-1-carbonyl)-1,5-diphenylpentan-3-ylcarbamate

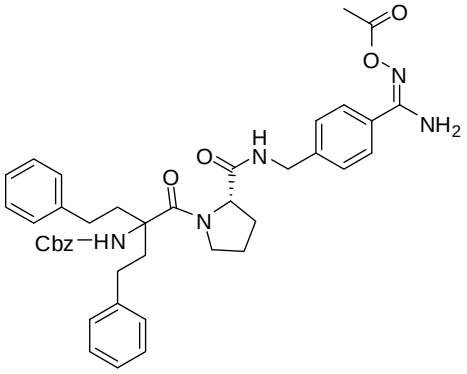
[Cbz-Dpheg-Pro-(4-OxAm)benzylamide]



120 mg (0.191 mmol) of the nitrile **201** were converted into hydroxyamidine according to method O and used for the synthesis of intermediate **203** without further purification.
Yield: not determined, brown oil.
HPLC: 54.4% B (purity: 50%).

203) (*S*)-benzyl 3-(2-(4-(*N'*-acetoxycarbamimidoyl)benzylcarbamoyl)-pyrrolidine-1-carbonyl)-1,5-diphenylpentan-3-ylcarbamate

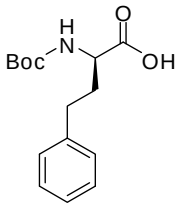
[Cbz-Dpheg-Pro-(4-AcOxAm)benzylamide]



0.191 mmol intermediate **202** were acetylated according to method P and used for the synthesis of inhibitor **MI-0458** without further purification.
Yield: not determined, brown oil.
HPLC: 65.1% B (purity: 35%).

204) (*R*)-2-(*tert*-butoxycarbonylamino)-4-phenylbutanoic acid

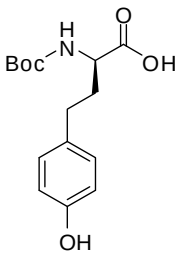
[Boc-D-hPhe-OH]



100 mg (0.558 mmol) H-D-hPhe-OH were Boc-protected according to method A.
Yield: 150 mg (0.537 mmol; 96.2%), white solid.
HPLC: 53.1% B (purity: 96%).

205) (*R*)-2-(*tert*-butoxycarbonylamino)-4-(4-hydroxyphenyl)butanoic acid

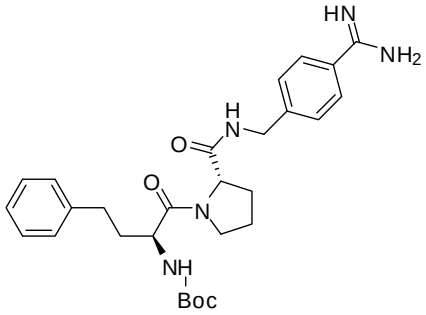
[Boc-D-hTyr-OH]



100 mg (0.364 mmol) H-D-hTyr-OH × HBr (Iris) were Boc-protected according to method A (HPLC control). An additional 19.7 mg (0.091mmol, 0.25 equiv) of di-*tert.*-butylpyrocarbonate were added at 0 °C and the mixture was stirred for additional 2 h.
Yield: 100 mg (0.339 mmol; 93%), white solid.
HPLC: 41.3% B (purity: 80%).

206) *tert*-butyl (*S*)-1-((*S*)-2-(4-carbamimidoylbenzylcarbamoyl)pyrrolidin-1-yl)-1-oxo-4-phenylbutan-2-ylcarbamate

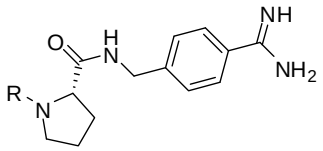
[Boc-hPhe-Pro-4-Amba]

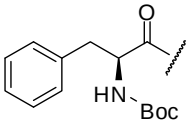
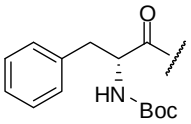
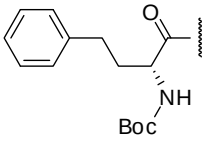
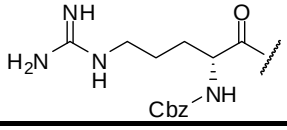


39.8 mg (0.142 mmol) Boc-hPhe-OH were coupled with 50 mg (0.157 mmol) intermediate **194** according to method I. The solvent was removed *in vacuo* and the residue was used for the synthesis of inhibitor **MI-0464** without further purification.
Yield: not determined, yellow oil.
HPLC: 42.3% B (purity: 70%).

Additional *N*-protected amino acids were coupled with 50 mg (0.157 mmol) intermediate **194** according to method I and provided analogues **207-210** (Table 5.15).

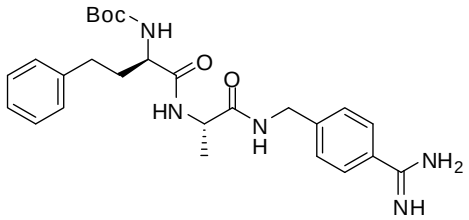
Table 5.15: Synthesis, yield and analytical characterization of compounds **205-208** having the general formula.



No.	R	Educts: mg/mmol	HPLC % B, purity %
207		Boc-Phe-OH 37.9/0.142	39.5, 89.3
208		Boc-D-Phe-OH 37.9/0.142	40.5, 90
209		Intermediate **204** Boc-D-hPhe-OH 39.8/0.142	43.5, 87.4
210		Cbz-D-Arg-OH × HCl 49.2/0.142	28.0, 79,3

211) *tert*-butyl (*R*)-1-((S)-1-(4-carbamimidoylbenzylamino)-1-oxopropan-2-ylamino)-1-oxo-4-phenylbutan-2-ylcarbamate

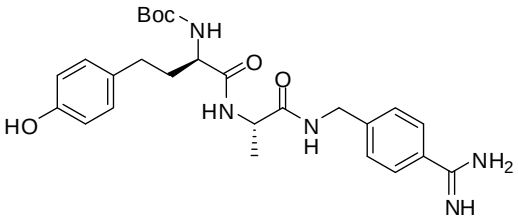
[Boc-D-hPhe-Ala-4-Amba]



57.6 mg (0.206 mmol) Boc-D-hPhe-OH (**204**) were coupled with 50 mg (0.227 mmol) H-Ala-4-Amba × 2 HCl (provided by Dr. Frank Sielaff) according to method I. The solvent was removed *in vacuo* and the residue was used for the synthesis of inhibitor **MI-0469** without further purification.
Yield: not determined, yellow oil.
HPLC: 41.02% B (purity: 81.8%).

212) *tert*-butyl (*R*)-1-((S)-1-(4-carbamimidoylbenzylamino)-1-oxopropan-2-ylamino)-4-(4-hydroxyphenyl)-1-oxobutan-2-ylcarbamate

[Boc-D-hTyr-Ala-4-Amba]



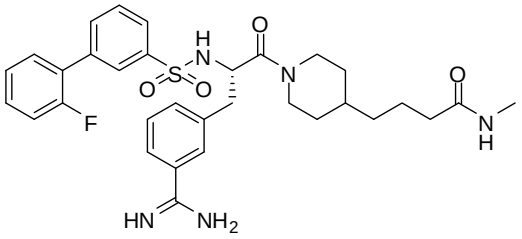
26.8 mg (0.09 mmol) Boc-D-hTyr-OH (**205**) were coupled with 22 mg (0.099 mmol) H-Ala-4-Amba × 2 HCl according to method I. The solvent was removed *in vacuo* and the residue was used for the synthesis of inhibitor **MI-0470** without further purification.
Yield: not determined, yellow oil.
HPLC: 41.02% B (purity: 55%).

5.5 Synthesis of inhibitors

213) (*S*)-4-(1-(3-(3-carbamimidoylphenyl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)-propanoyl)piperidin-4-yl)-*N*-methylbutanamide × TFA

[2′F-biphenyl-3-sulfonyl-Phe(3-Am)-4-Pip-*N*-methylbutanamide × TFA]

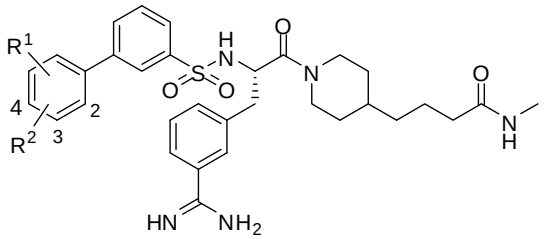
(MI-0403)



The intermediate **22** was converted into the amidine according to method Q. The product was purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 23 mg (0.032 mmol), white powder.
HPLC: 45.3% B; TLC: R_f0.87; MS calcd: 607.26, found: 608.18 $(M+H)^+$.

Additional analogues **214-219** (Table 5.16) were prepared from intermediates **23-28** using method Q.

Table 5.16: Synthesis, yield and analytical characterization of compounds **214-219** having the general formula.

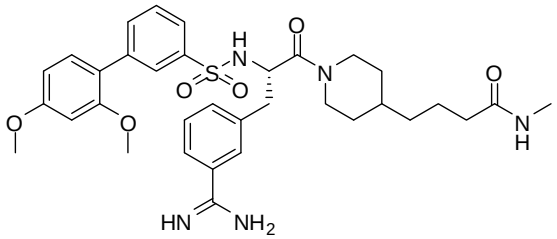


No.	R^1	R^2	Educt Intermediate No.	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
214 MI-0404	3-F	-	**23**	15/0.021	45.4	0.86	607.26, 608.23
215 MI-0405	4-F	-	**24**	29/0.04	44.6	0.85	607.26, 608.17
216 MI-0406	2-Cl	4-Cl	**25**	15/0.019	48.6	0.84	657.19, 658.16
217 MI-0440	2-Cl	3-Cl	**26**	17/0.022	48	0.85	657.19, 658.41
218 MI-0442	3-Cl	4-Cl	**27**	26/0.034	48.7	0.85	657.19, 658.41
219 MI-0412	2-Cl	4-EtO	**28**	15/0.019	50.06	0.78	667.26, 668.38

[a]All final inhibitors were obtained as white powder after lyophilization.

220) (*S*)-4-(1-(3-(3-carbamimidoylphenyl)-2-(2',4'-dimethoxybiphenyl-3-ylsulfonamido)propanoyl)piperidin-4-yl)-N-methylbutanamide × TFA

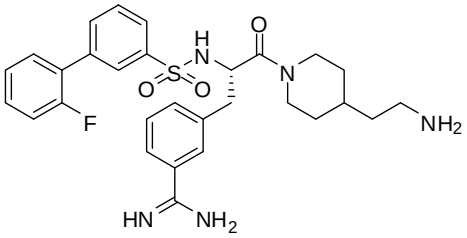
[2′,4′dimethoxybiphenyl-3-sulfonyl-Phe(3-Am)-4-Pip-*N*-methylbutanamide × TFA] **(MI-0438)**



The crude intermediate **29** were hydrogenated according to method F, the product was purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 14 mg (0.018 mmol), white powder.
HPLC: 43.9% B; TLC: R_f 0.83; MS calcd: 649.29, found: 650.39 $(M+H)^+$.

221) (*S*)-3-(3-(4-(2-aminoethyl)piperidin-1-yl)-2-(2'-fluorobiphenyl-3-ylsulfonamido)-3-oxopropyl)benzimidamide × 2 TFA

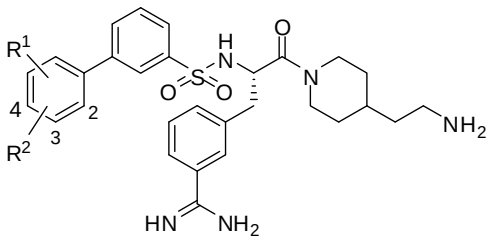
[2′F-biphenyl-3-sulfonyl-Phe(3-Am)-4-Pip-ethylamine × 2 TFA] **(MI-0433)**



The crude intermediate **55** was deprotected according to method B.
Yield: 17 mg (0.022 mmol), white powder.
HPLC: 24% B; TLC: R_f 0.73; MS calcd: 551.24, MS found: 552.29 $(M+H)^+$.

Additional analogues **222-228** (Table 5.17) were prepared from intermediates **56-62** using method B.

Table 5.17: Synthesis, yield, and analytical characterization of compounds **222-228** having the general formula.

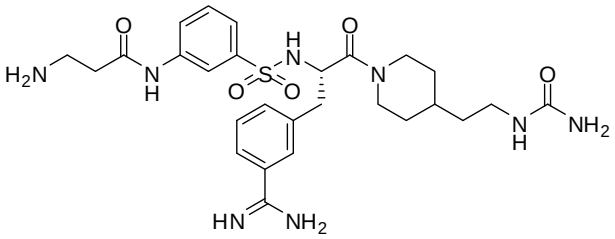


No.	R^1	R^2	Educt Intermediate No.	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
222 MI-0431	3-F	-	**56**	18/0.023	34.3	0.72	551.24, 552.31
223 MI-0434	4-F	-	**57**	12/0.015	34.6	0.73	551.24, 552.22
224 MI-0432	2-Cl	4-Cl	**58**	18/0.022	39.4	0.74	601.17, 602.21
225 MI-0441	2-Cl	3-Cl	**59**	13/0.016	39.7	0.74	601.17, 602.11
226 MI-0443	3-Cl	4-Cl	**60**	15/0.018	40.8	0.75	601.27, 602.10
227 MI-0413	2-Cl	4-EtO	**61**	14/0.019	41	0.75	611.23, 612.32
228 MI-0439	2-MeO	4-MeO	**62**	20/0.024	36.1	0.71	593.27, 594.41

[a]All final inhibitors were obtained as white powder after lyophilization.

229) (*S*)-3-amino-*N*-(3-(*N*-(3-(3-carbamimidoylphenyl)-1-oxo-1-(4-(2-ureidoethyl)-piperidin-1-yl)propan-2-yl)sulfamoyl)phenyl)propanamide × 2 TFA

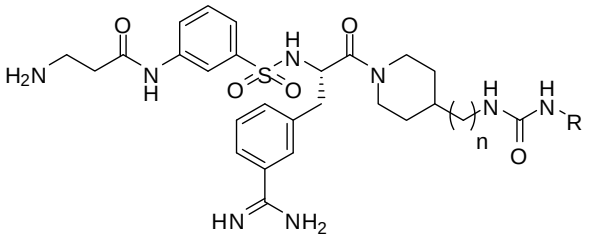
[H-*β*Ala-3-NH-phenylsulfonyl-Phe(3-Am)-4-Pip-Et-urea × 2 TFA] **(MI-0449)**

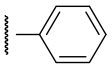
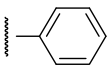
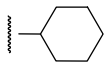
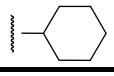


0.057 mmol intermediate **111** were hydrogenated according to method F.
Yield: 12 mg (0.015 mmol), white powder.
HPLC: 23.4% B; TLC: R_f 0.55; MS calcd: 586.27, found: 587.41 $(M+H)^+$.

Additional analogues **230-238** (Table 5.18) were prepared by hydrogenation of the intermediates **112-120** using method F.

Table 5.18: Synthesis, yield and analytical characterization of compounds **230-238** having the general formula.

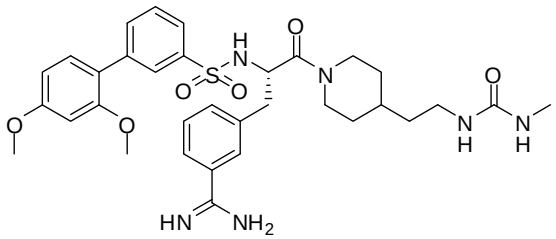


No.	n	R	Educt Intermediate No.	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
230 MI-0451	0	H	**112**	13/0.017	20.3	0.54	558.24, 559.31
231 MI-0436	2	Me	**113**	13/0.016	24.4	0.63	600.28, 601.22
232 MI-0450	0	Me	**114**	12/0.015	21.3	0.59	572.25, 573.30
233 MI-0437	2	Et	**115**	14/0.017	26.2	0.66	614.27, 615.41
234 MI-0452	0	Et	**116**	19/0.023	23.7	0.59	586.27, 587.31
235 MI-0444	2		**117**	15/0.017	32.2	0.67	662.27, 663.40
236 MI-0454	0		**118**	17/0.020	30.4	0.71	634.27, 635.61
237 MI-0445	2		**119**	20/0.022	34.1	0.67	668.35, 669.51
238 MI-0453	0		**120**	19/0.022	31.7	0.65	640.35, 641.32

[a]white powder after lyophilization

239) (*S*)-3-(2-(2',4'-dimethoxybiphenyl-3-ylsulfonamido)-3-(4-(2-(3-methylureido)-ethyl)piperidin-1-yl)-3-oxopropyl)benzimidamide × TFA

[2′,4′-Dimethoxybiphenyl-3-sulfonyl-Phe(3-Am)-4-Pip-Et-methylurea × TFA] **(MI-0460)**



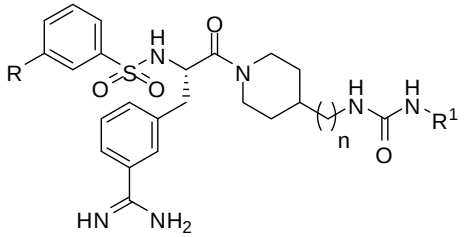
0.064 mmol intermediate **123** was reduced by zinc dust in 90% HOAc according to method Q.

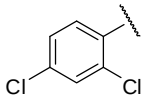
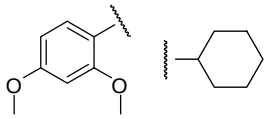
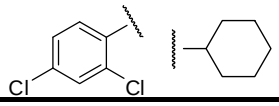
Yield: 14 mg (0.018 mmol), white powder.

HPLC: 41.4% B; TLC: R_f 0.84; MS calcd: 650.29, found: 651.42 $(M+H)^+$.

Additional analogues **240**-**242** (Table 5.19) were prepared by reduction of intermediates **124**-**126** using method Q.

Table 5.19: Synthesis, yield and analytical characterization of compounds **240**-**242** having the general formula.

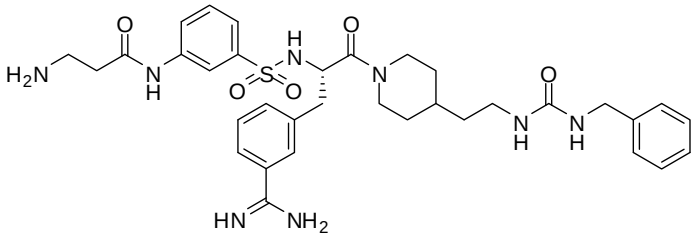


No.	n	R	R^1	Educt Intermediate No.	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
240 MI-0461	2		Me	**124**	13/0.017	46.4	0.85	658.19, 659.37
241 MI-0462	0			**125**	25/0.031	48.7	0.85	690.32, 691.51
242 MI-0463	0			**126**	13/0.016	53.5	0.87	698.22, 700.28

[a]slight yellowish powder after lyophilization

243) (*S*)-3-amino-*N*-(3-(*N*-(1-(4-(2-(3-benzylureido)ethyl)piperidin-1-yl)-3-(3-carbamimidoylphenyl)-1-oxopropan-2-yl)sulfamoyl)phenyl)propanamide × 2 TFA

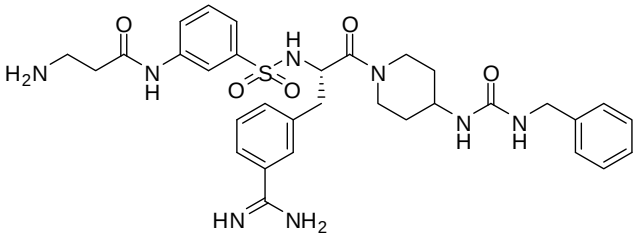
[H-*β*Ala-3-NH-phenylsulfonyl-Phe(3-Am)-4-Pip-Et-Bzlu × 2 TFA] **(MI-0446)**



The intermediate **127** was deprotected according to method E, purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 11 mg (0.012 mmol), white powder.
HPLC: 31.6% B; TLC: R_f 0.66; MS calcd: 676.32, found. 677.41 $(M+H)^+$.

244) (*S*)-3-amino-*N*-(3-(*N*-(1-(4-(3-benzylureido)piperidin-1-yl)-3-(3-carbamimidoylphenyl)-1-oxopropan-2-yl)sulfamoyl)phenyl)propanamide × 2 TFA

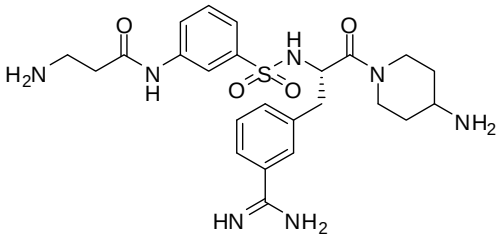
[H-*β*Ala-3-NH-phenylsulfonyl-Phe(3-Am)-4-Pip-Bzlu × 2 TFA] **(MI-0455)**



The intermediate **128** was deprotected as described for the synthesis of inhibitor **MI-0446** (method E).
Yield: 14 mg (0.016 mmol), white powder.
HPLC: 30.1% B; TLC: R_f 0.71; MS calcd: 648.28, found: 649.71 $(M+H)^+$.

245) (*S*)-3-amino-*N*-(3-(*N*-(1-(4-aminopiperidin-1-yl)-3-(3-carbamimidoylphenyl)-1-oxopropan-2-yl)sulfamoyl)phenyl)propanamide × 3 TFA

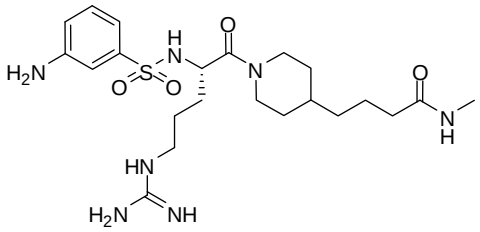
[H-*β*Ala-3-NH-phenylsulfonyl-Phe(3-Am)-4-Pip-NH_2 × 3 TFA] **(MI-0458)**



The intermediate **130** were hydrogenated according to method F. The solvent was removed *in vacuo*, the product was purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 40mg (0.047), white powder.
HPLC: 17.5% B; TLC: R_f 0.54; MS calcd: 515.23, found: 516.31 $(M+H)^+$.

246) (*S*)-4-(1-(2-(3-aminophenylsulfonamido)-5-guanidinopentanoyl)piperidin-4-yl)-*N*-methylbutanamide × 2 TFA

[3-NH_2-phenylsulfonyl-Arg-4-Pip-*N*-methylbutanamide × 2 TFA] **(MI-0401)**

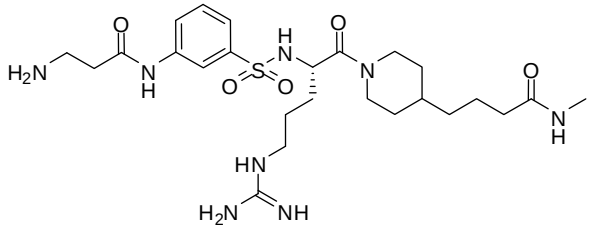


The crude intermediate **133** was deprotected using 90% TFA. The mixture was stirred for 2 h. The solvent was removed *in vacuo*, the product was purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 45 mg (0.622 mmol), white powder.
HPLC: 24.9% B; TLC: R_f 0.75; MS calcd: 495.26, found: 496.2 $(M+H)^+$.

247) (*S*)-4-(1-(2-(3-(3-aminopropanamido)phenylsulfonamido)-5-guanidino-pentanoyl)piperidin-4-yl)-*N*-methylbutanamide × 2 TFA

[H-*β*Ala-3-NH-phenylsulfonyl-Arg-4-Pip-*N*-methylbutanamide × 2 TFA]

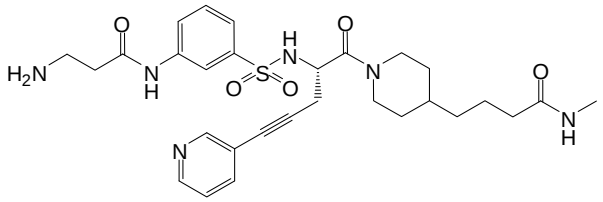
(MI-0402)



The crude intermediate **134** was deprotected and purified as described for the synthesis of inhibitor **246**.
Yield: 68 mg (0.086 mmol), white powder.
HPLC: 24.1% B; TLC: R_f 0.68; MS calcd: 566.30, found: 567.21 $(M+H)^+$.

248) (*S*)-4-(1-(2-(3-(3-aminopropanamido)phenylsulfonamido)-5-(pyridin-3-yl)pent-4-ynoyl)piperidin-4-yl)-*N*-methylbutanamide × 2 TFA

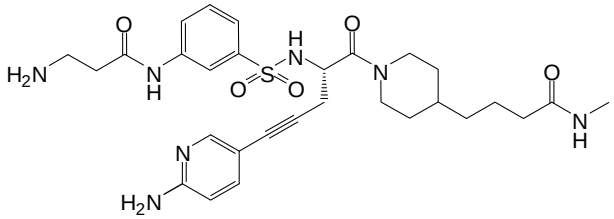
[H-*β*Ala-3-NH-phenylsulfonyl-Pra-5-(pyridin-3-yl)-4-Pip-*N*-methylbutanamide × 2 TFA] **(MI-0407)**



The crude intermediate **140** was deprotected according to method H, the product was purified by preparative HPLC and lyophilized from 80 % *tert*-butanol.
Yield: 7 mg (0.009 mmol), slight yellowish powder.
HPLC: 27.6% B; TLC: R_f 0.77; MS calcd: 582.26, found: 583.1 $(M+H)^+$.

249) (*S*)-4-(1-(2-(3-(3-aminopropanamido)phenylsulfonamido)-5-(6-aminopyridin-3-yl)pent-4-ynoyl)piperidin-4-yl)-*N*-methylbutanamide × 2 TFA

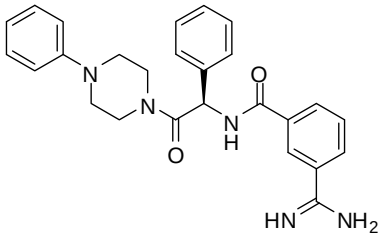
[H-*β*Ala-3-NH-phenylsulfonyl-Pra-5-(6-aminopyridin-3-yl)-4-Pip-*N*-methyl-butanamide × 2 TFA] **(MI-0408)**



The Tfa-group of intermediate **143** was removed by 1 N NaOH according to method H.
Yield: 15 mg (0.018 mmol), slight yellowish powder.
HPLC: 26.6% B; TLC: R_f 0.56; MS calcd: 597.27, found: 598.22 $(M+H)^+$.

250) (*R*)-3-carbamimidoyl-*N*-(2-oxo-1-phenyl-2-(4-phenylpiperazin-1-yl)ethyl)-benzamide × TFA

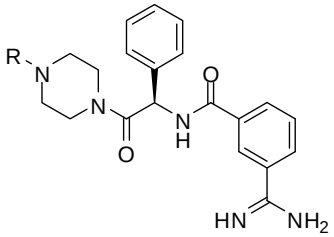
[(3-Am)benzoyl-D-Phg-4-phenylpiperazin-1-yl × TFA] **(MI-0415)**

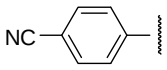
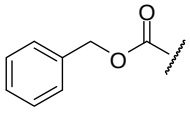
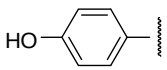
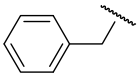


50 mg (0.121 mmol) intermediate **149** were coupled with 19.6 mg (0.121 mmol) 1-phenylpiperazine according to method I. The product was purified by preparative HPLC and lyophilized from 80 % *tert*-butanol.
Yield: 30 mg (0.054 mmol), white powder.
HPLC: 38.2% B; TLC: R_f 0.81; MS calcd: 441.22, found: 442.4 $(M+H)^+$.

Additional analogues **251-254** (Table 5.20) were prepared by coupling 50 mg (0.121 mmol) intermediate **149** with (0.121 mmol, 1.0 equiv) of various piperazine derivatives using method I.

Table 5.20: Synthesis, yield and analytical characterization of inhibitors **251-254** having the general formula.

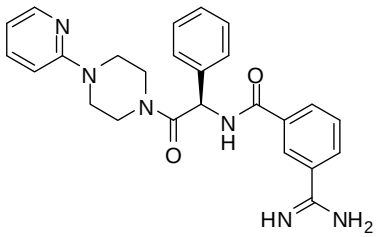


No.	R	Educt piperazine derivative mg	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
251 MI-0418		4-(piperazin-1-yl)benzonitrile 22.7	21/0.037	41.2	0.80	466.21, 467.41
252 MI-0420		1-Cbz-piperazine 26.6	13/0.021	43.7	0.85	499.22, 500.01
253 MI-0421		1-(4-hydroxy-phenyl)-piperazine 21.6	25/0.044	25.6	0.81	457.20, 458.30
254 MI-0426		1-benzyl-piperazine 21.3	17/0.03	29.1	0.76	455.23, 456.22

[a]white powder after lyophilization

255) (*R*)-3-carbamimidoyl-*N*-(2-oxo-1-phenyl-2-(4-(pyridin-2-yl)piperazin-1-yl)-ethyl)benzamide × 2 TFA

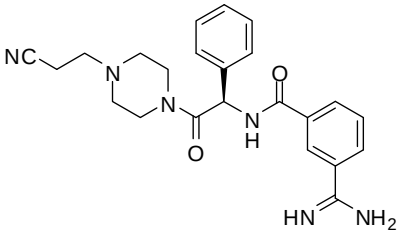
[(3-Am)benzoyl-D-Phg-4-(pyridin-2-yl)piperazin-1-yl × 2 TFA] **(MI-0422)**



50 mg (0.121 mmol) intermediate **149** were coupled with 19.3 μl (0.121 mmol) 1-(2-Pyr)piperazine according to method J, and the product was purified by preparative HPLC and lyophilized from water.
Yield: 30 mg (0.045 mmol), slight yellowish powder.
HPLC: 23.9% B; TLC: R_f 0.79; MS calcd: 442.21, found: 443.22 $(M+H)^+$.

256) (*R*)-3-carbamimidoyl-*N*-(2-(4-(2-cyanoethyl)piperazin-1-yl)-2-oxo-1-phenyl-ethyl)benzamide × 2 TFA

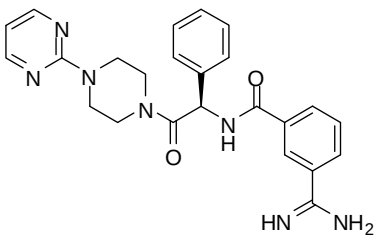
[(3-Am)benzoyl-D-Phg-4-(CN-Et)piperazin-1-yl × 2 TFA] **(MI-0423)**



50 mg (0.121 mmol) intermediate **149** were coupled with 16.8 mg (0.121 mmol) 3-piperazinopropionitrile as described for the synthesis of compound **255** (method J).
Yield: 15 mg (0.023 mmol), slight yellowish powder.
HPLC: 20.5% B; TLC: R_f 0.79; MS calcd: 418.21, found: 419.41 $(M+H)^+$.

257) (*R*)-3-carbamimidoyl-*N*-(2-oxo-1-phenyl-2-(4-(pyrimidin-2-yl)piperazin-1-yl)-ethyl)benzamide × TFA

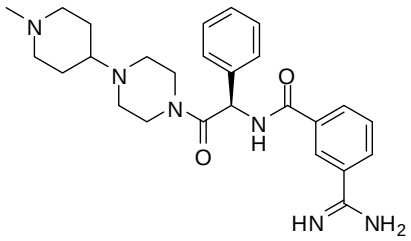
[(3-Am)benzoyl-D-Phg-4-(pyrimidin-2-yl)piperazin-1-yl × TFA] **(MI-0427)**



The product **257** (**MI-0427**) was prepared from 50 mg (0.121 mmol) intermediate **149** and 19.9 mg (0.121 mmol) 2-(piperazin-1-yl)pyrimidine as described for the synthesis of compound **255** (method J).
Yield: 10 mg (0.018 mmol), yellowish powder.
HPLC: 31.1% B; TLC: R_f 0.79; MS calcd: 443.21, found: 444.11 $(M+H)^+$.

258) (*R*)-3-carbamimidoyl-*N*-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)benzamide × 3 TFA

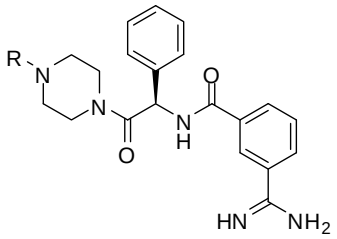
[(3-Am)benzoyl-D-Phg-4-(1-Me-Pip)piperazin-1-yl × 3 TFA] **(MI-0416)**

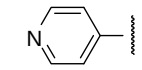
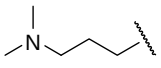
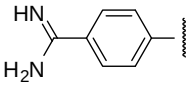


The intermediate **164** was hydrogenated according to method method F.
Yield: 23 mg (0.029 mmol), white powder.
HPLC: 17.1% B; TLC: R_f 0.71; MS calcd: 462.27, found: 463.41 $(M+H)^+$.

Additional analogues **259**-**261** (Table 5.21) were prepared from intermediates **165**-**167** by hydrogenation according to method F.

Table 5.21: Synthesis, yield and analytical characterization of inhibitors **259**-**261** having the general formula.

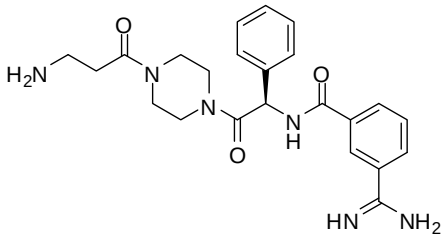


No.	R	Educt Intermediate No.	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
259 MI-0417		**165**	23/0.034	23.2	0.71	442.21, 443.51
260 MI-0424		**166**	30/0.038	21.8	0.70	450.27, 451.32
261 MI-0419		**167**	32/0.045	28.1	0.76	483.24, 484.21

[a]All final inhibitors were obtained as white powder after lyophilization.

262) (*R*)-*N*-(2-(4-(3-aminopropanoyl)piperazin-1-yl)-2-oxo-1-phenylethyl)-3-carbamimidoylbenzamide × 2 TFA

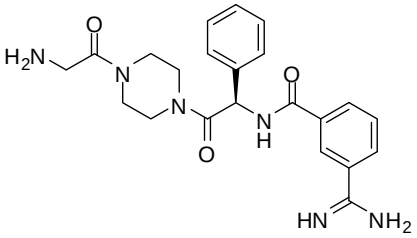
[(3-Am)benzoyl-D-Phg-4-(β-Ala)piperazin-1-yl × 2 TFA] **(MI-0429)**



The intermediate **170** was deprotected by TFA according to method B.
Yield: 55 mg (0.083 mmol), white powder.
HPLC: 20.2% B; TLC: R_f 0.64; MS calcd: 436.22, found: 437.28 $(M+H)^+$.

263) (*R*)-*N*-(2-(4-(2-aminoacetyl)piperazin-1-yl)-2-oxo-1-phenylethyl)-3-carbamimidoylbenzamide × 2 TFA

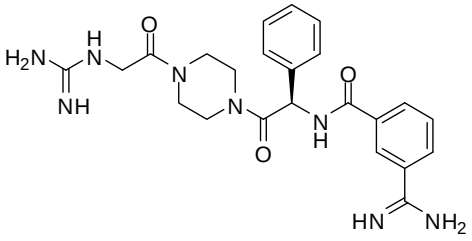
[(3-Am)benzoyl-D-Phg-4-(Gly)piperazin-1-yl × 2 TFA] **(MI-0430)**



The intermediate **171** were deprotected by TFA according to the method B.
Yield: 55 mg (0.085 mmol), white powder.
HPLC: 19.1% B; TLC: R_f 0.62; MS calcd: 422.21, found: 423.23 $(M+H)^+$.

264) (*R*)-3-carbamimidoyl-*N*-(2-(4-(2-guanidinoacetyl)piperazin-1-yl)-2-oxo-1-phenylethyl)benzamide × 2 TFA

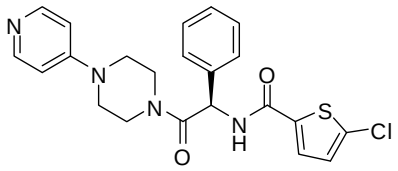
[(3-Am)benzoyl-D-Phg-4-(2-guanidinoacetyl)piperazin-1-yl × 2 TFA] **(MI-0435)**



25 mg (0.038 mmol) of inhibitor **263** were converted into the analogous guanidine derivative according to method S.
Yield: 25 mg (0.036 mmol), white powder.
HPLC: 20.8% B; TLC: R_f 0.71; MS calcd: 464.23, found: 465.37 $(M+H)^+$.

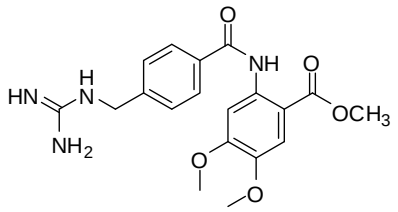
265) (*R*)-5-chloro-*N*-(2-oxo-1-phenyl-2-(4-(pyridin-4-yl)piperazin-1-yl)ethyl)-thiophene-2-carboxamide × TFA

[5-Cl-thiophene-2-carboxyl-D-Phg-4-(pyridin-4-yl)piperazin-1-yl × TFA] **(MI-0423)**



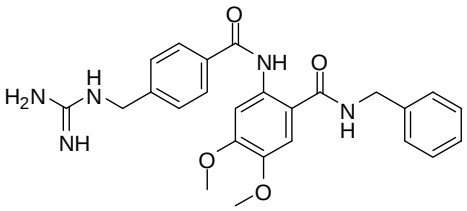
50mg (0.169 mmol) intermediate **173** were coupled with 27.59 mg (0.169 mmol) 1-(pyridin-4-yl)piperazine according to method L.
Yield: 15 mg (0.027 mmol), white powder.
HPLC: 41.8% B; TLC: R_f 0.92; MS calcd: 440.11, found: 441.72 $(M+H)^+$.

266) Methyl 2-(4-(guanidinomethyl)benzamido)-4,5-dimethoxybenzoate × TFA (MI-0409)



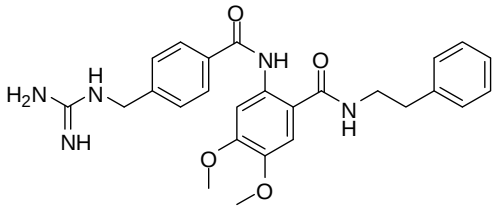
60 mg of the amine intermediate **177** were converted into the guanidine according to method S.
Yield: 52.2 mg (0.123 mmol), white powder.
HPLC: 40.3% B; TLC: R_f 0.92; MS calcd: 386.16, found. 387 $(M+H)^+$.

267) *N*-benzyl-2-(4-(guanidinomethyl)benzamido)-4,5-dimethoxybenzamide × TFA (MI-0410)



90 mg of the amine intermediate **179** were converted into the guanidine according to method S.
Yield: 105 mg (0.182 mmol), white powder.
HPLC: 43.1% B; TLC: R_f 0.84; MS calcd: 461.21, found: 462.22 $(M+H)^+$.

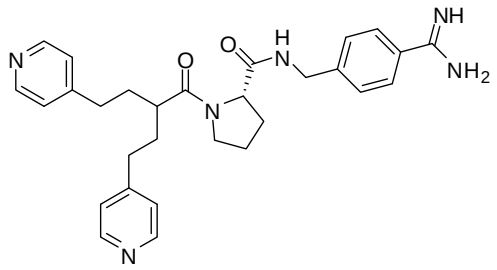
268) 2-(4-(Guanidinomethyl)benzamido)-4,5-dimethoxy-*N*-phenethylbenzamide × TFA (MI-0411)



70 mg of the amine **181** were converted into the guanidine according to method S.
Yield: 21 mg (0.036 mmol), white powder.
HPLC: 45.2 % B; TLC: R_f 0.86; MS calcd: 475.22, found: 476.21 $(M+H)^+$.

269) (*S*)-*N*-(4-carbamimidoylbenzyl)-1-(4-(pyridin-4-yl)-2-(2-(pyridin-4-yl)ethyl)-butanoyl)pyrrolidine-2-carboxamide × 3 TFA

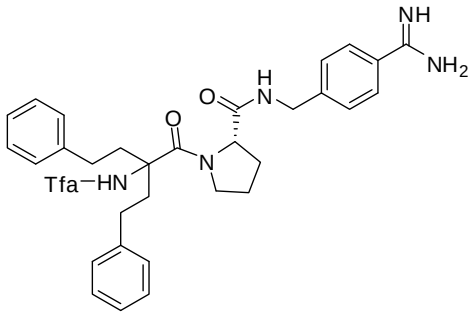
[4-(Pyridin-4-yl)-2-(2-(pyridin-4-yl)ethyl)butanoyl-)-Pro-(4-Amba) × 3 TFA] **(MI-0400)**



100 mg (0.201 mmol) intermediate **183** were coupled with 70.6 mg (0.221 mmol) intermediate **194** according to method I.
Yield: 115 mg (0.137 mmol), white powder.
HPLC: 16.5% B; TLC: R_f 0.6; MS calcd: 498.27, found: 499.25 $(M+H)^+$.

270) (*S*)-*N*-(4-carbamimidoylbenzyl)-1-(2-phenethyl-4-phenyl-2-(2,2,2-trifluoro-acetamido)butanoyl)pyrrolidine-2-carboxamide × TFA

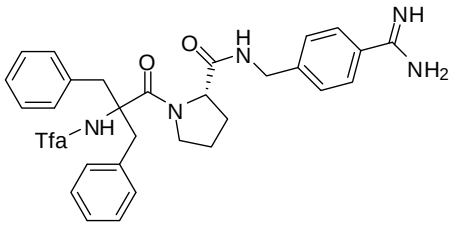
[Tfa-Dpheg-Pro-(4-Amba) × TFA] **(MI-0457)**



The intermediate **197** was hydrogenated according to method F.
Yield: 69 mg (0.096 mmol), white powder.
HPLC: 53.2% B; TLC: R_f 0.88; MS calcd: 607.28, found: 608.31, $(M+H)^+$.

271) (*S*)-1-(2-benzyl-3-phenyl-2-(2,2,2-trifluoroacetamido)propanoyl)-*N*-(4-carbamimidoylbenzyl)pyrrolidine-2-carboxamide × TFA

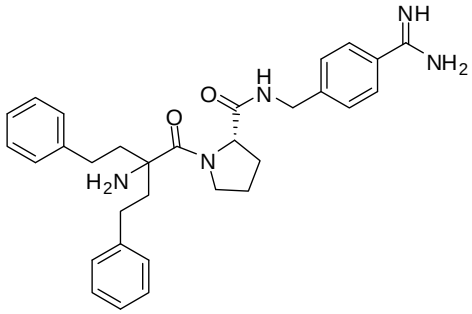
[Tfa-Dbzg-Pro-(4-Amba) × TFA] **(MI-0456)**



The intermediate **200** was hydrogenated according to method F.
Yield: 133 mg (0.192 mmol), white powder.
HPLC: 48.3% B; TLC: R_f 0.83; MS calcd: 579.25, found. 580.22 $(M+H)^+$.

272) (*S*)-1-(2-amino-2-phenethyl-4-phenylbutanoyl)-*N*-(4-carbamimidoylbenzyl)-pyrrolidine-2-carboxamide × 2 TFA

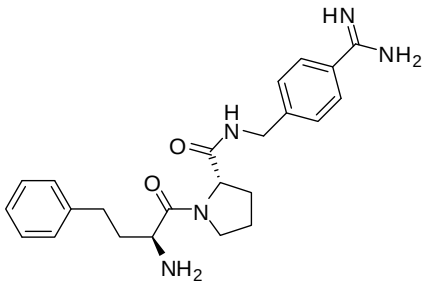
[H-Dpheg-Pro-(4-Amba) × 2 TFA] **(MI-0459)**



The intermediate **203** was hydrogenated according to method F.
Yield: 57 mg (0.077 mmol), white powder.
HPLC: 37.7% B; TLC: R_f 0.81; MS calcd: 511.29, found. 512.28 $(M+H)^+$.

273) (*S*)-1-((*S*)-2-amino-4-phenylbutanoyl)-*N*-(4-carbamimidoylbenzyl)-pyrrolidine-2-carboxamide × 2 TFA

[H-hPhe-Pro-4-Amba × 2 TFA] **(MI-0464)**

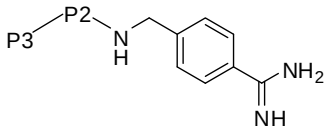


(*S*)-1-((*S*)-2-amino-4-phenylbutanoyl)-*N*-(4-carbamimidoylbenzyl)pyrrolidine-2-carboxamide

The intermediate **206** was deprotected by TFA according to method B.
Yield: 35 mg (0.055 mmol), white powder.
HPLC: 25.1% B; TLC: R_f 0.75; MS calcd: 407.23, found: 408.29 $(M+H)^+$.

Additional analogues **274-278** (Table 5.22) were prepared from deprotecting the intermediates **207-209, 211, 212** according to method B.

Table 5.22: Synthesis, yield and analytical characterization of inhibitors **274-278** having the general formula.

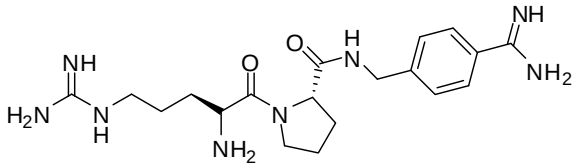


No.	P3	P2	Educt Intermediate No.	Yield[a] mg/mmol	HPLC % B	TLC R_f	MS calcd, found $(M+H)^+$
274 MI-0465	H-Phe	Pro	**207**	53/085	22.3	0.74	393.22, 394.12
275 MI-0466	H-D-Phe	Pro	**208**	39/0.63	23.6	0.77	393.22, 394.29
276 MI-0467	H-D-hPhe	Pro	**209**	35/0.055	26.3	0.79	407.23, 408.37
277 MI-0469	H-D-hPhe	Ala	**211**	45/0.074	24.8	0.75	381.22, 382.43
278 MI-0470	H-D-hTyr	Ala	**212**	20/0.032	18.9	0.72	397.22, 398.22

[a]white powder after lyophilization

279) (*S*)-1-((*R*)-2-amino-5-guanidinopentanoyl)-*N*-(4-carbamimidoylbenzyl)-pyrrolidine-2-carboxamide × 3 TFA

[H-D-Arg-Pro-4-Amba × 3 TFA] **(MI-0468)**



Intermediate **210** was deprotected according to method E, purified by preparative HPLC and lyophilized from 80% *tert*-butanol.
Yield: 49 mg (0.066 mmol), white powder.
HPLC: 14.6% B; TLC: R_f 0.2; MS calcd: 402.25, found. 403.51 $(M+H)^+$.

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Publications

A part of this work has been published

M. Hammami, E. Rühmann, E. Maurer, A. Heine, M. Gütschow, G. Klebe, T. Steinmetzer; New 3-amidinophenylalanine derived inhibitors of matriptase. *MedChemComm***,** 2012(3), 807-813.

M. T. Sisay, T. Steinmetzer, M. Stirnberg, E. Maurer, M. Hammami, Jürgen Bajorath, and M. Gütschow, Identification of the First Low-Molecular-Weight Inhibitors of Matriptase-2. J. Med. Chem. 2010, 53.

Steinmetzer T., Meyer D., Hammami M., Sielaff F., Böttcher-Friebertshäuser E., Garten W.; Verwendung von Hemmstoffen der TMPRSS2 als Arzneimittel.
Patent No. DE 10 2011 108 346.8.

Posters:

"Synthesis and characterization of new matriptase inhibitors – Potential drugs for the treatment of cancer";
Poster presentation *Synthesis and characterization of new matriptase inhibitors*
10th German Peptide Symposium, Berlin; März 2011.

Acknowledgement

I would like to give my sincere appreciation to my supervisor Prof. Dr. Torsten Steinmetzer for his generous support during the entire time of my project, his scientific foresight and discussions, direct guidance, sagacity, and always positive personality. Because of his safeguard, I did not lose my confidence toward my Ph.D. degree even at hardest time. I would like to thank him for his time spent with me and his efforts in all revisions of the manuscript.

My sincere appreciation and gratefulness go to Mr. Yousef Jameel our spiritual father for his kindness, generosity, and his financial support of my project and study period in Germany. Special thanks are to Mrs. Petra Kienle and Mrs. Miram Groß, of the international office in Philipps University, for all their care and concern; I have had since the first moment of my residence and Ph.D in Marburg

I would like to give special thanks to all my committee members; Prof. Michael Gütschow, Prof. Carsten Culmsee and Prof. Klaus Kuschinsky for their scientific guidance, encouragement and support. My kind regards goes to Prof. Michael Gütshcow, for his willingness to be my second reviewer and for the excellent cooperation in the testing of inhibitors with matriptase-2.
I would like to thank Dr. Eva Böttcher Friebertshäuser and Garten's group for the great cooperation and the rapid testing of inhibitors in cell culture, and to thank Eggert Rühmann from Klebe's group for the crystal structure of thrombin in complex with inhibitors.

I thank my all fellow labmates, the former and current members, I have met in our Steinmetzer's group; Daniela Meyer, Heike Lang-Henkel, Kornelia Hardes, Dr. Frank Sielaff, Dr. Gero Becker, Sebastian Saupe, my brother Zouhir Hammamy, Alexander Maiwald and Wegderes Endreas, for the stimulating discussions, their friendship and family atmosphere. Many thanks for all the fun we have had in the last four years inside and outside work time. Special thanks are for my officemate Sebastian for all his help during my project time, Daniela for HAT enzyme kinetics measurements and Kornelia for revision of the manuscript and all you have done to me.

I would like to thank Heike Lang-Henkel and Nina Zitzer for all the mass spectrometer measurements done by them.

Last but not the least; my sincere thanks and appreciation are to my lovely family; my parents, my sister Dima and my brother Zouhir for all unconditional love, spiritual support, and sucrifies throughout my life. Thanks that you were always by my side all time pushing me forward in every step of my life to come over all difficulties. My love and thanks are for you, my young friend and son Salah, who was by my side supporting and understanding the occupation in my project time.

Erklärung

Ich versichere, dass ich meine Dissertation

„Development of new inhibitors for the type II transmembrane serine protease matriptase“

selbständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 14. Juli 2012

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Curriculum Vitae

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