Development of Selective Nitrile Inhibitors and ‘Activity-Based’ Probes For Human Cathepsins K and S

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät
der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Maxim Frizler

aus

Kamyschnoje, Kasachstan

Bonn 2012
Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn.

1. Referent: Herr Professor Dr. Michael Gütschow
2. Referent: Frau Professor Dr. Christa E. Müller

Tag der Promotion: 14.05.2012
Erscheinungsjahr: 2012
Meiner Familie und Friederike
in Dankbarkeit
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ABBREVIATIONS

ABP  ‘activity-based’ probe
Ac   acetyl
AMC  7-amino-4-methylcoumarin
APP  amyloid precursor protein
BACE1 β-site APP-cleaving enzyme
Bn   benzyl
Boc  tert-butyloxy carbonyl
BODIPY boron dipyrromethene (difluoride)
Cath cathepsin
Cbz (Z) carboxy benzyl
CD   cluster of differentiation
CDI  1,1’-carbonyldiimidazole
CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHMP committee for medicinal products for human use
CLIP class II-associated invariant chain peptide
cmpd compound
d   doublet
DAD  diode array detector
dd  double doublet
DIPEA N,N-diisopropylethylamine
DMAP dimethylaminopyridine
DMF  dimethylformamide
DMSO dimethyl sulfoxide
DPP-IV dipeptidyl peptidase IV
dt  double triplet
DTT  dithiothreitol
EDC  1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA ethylenediaminetetraacetic acid
EI   electron ionization (electron impact)
ESI  electrospray ionization
Et   ethyl
GFP  green fluorescent protein
GLP  glucagon-like peptide
HBTU  2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate
HOBT  1-hydroxybenzotriazole
HPLC  high performance liquid chromatography
HSAB  hard and soft acids and bases
i-Bu  isobutyl
IFN  interferon
IL  interleukin
i-Pr  isopropyl
IR  infrared spectroscopy
m  multiplet
MCPBA  meta-chloroperoxybenzoic acid
Me  methyl
MHC  major histocompatibility complex
MS  mass spectrometry
NMM  N-methylmorpholine
NMR  nuclear magnetic resonance
pNA  para-nitroaniline/para-nitroanilide
RANK  receptor activator of nuclear factor-κB
rt  room temperature
s  singlet
SARs  structure-activity relationships
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
sept  septet
SERM  selective oestrogen receptor modulator
t  triplet
t-Bu  tert-butyl
TEA  triethylamine
TFA  trifluoroacetic acid
THF  tetrahydrofuran
TNF  tumor necrosis factor
tert  tertiary
UV  ultraviolet
VIS  visible
1. INTRODUCTION
1.1. GENERAL REMARKS

The human genome encodes more than 500 proteases (for a review, see [1]). Among them, a heterogeneous group is referred to as cathepsins, regardless of the catalytic mechanism. Cathepsins were described initially as lysosomal proteolytic enzymes due to their localization in the lysosomes. Whereas cathepsins A and G are serine proteases, and cathepsins D and E are aspartic proteases, cathepsins B, C, F, H, K, L, O, S, V, W and X are cysteine proteases of the papain-like subfamily C1A and represent the largest and best characterized group of cathepsins. Cysteine proteases of the C1A subfamily are ubiquitous among living organisms including bacteria, viruses, plants, lower and higher animals. Most of the cysteine cathepsins are endopeptidases, with the exception of the true exopeptidases cathepsins C and X. The main function of the eleven papain-like lysosomal cysteine proteases listed above is the degradation of proteins that have entered the lysosomal system. The cleavage of the peptide bond, catalyzed by cysteine cathepsins, is initiated by the nucleophilic attack of the active site cysteine to form an acyl enzyme and to release the first product. In the second step of the acyl transfer, the hydrolysis of the thiol ester bond leads to the release of the second product and to the formation of the free enzyme. The proteolytic activity is mediated by a catalytic triad composed of the cysteine residue located at the centre of the catalytic site, a histidine residue, and an asparagine residue for keeping the imidazole ring of histidine in a favourable orientation to the thiol group of the catalytic cysteine [2].

Cysteine cathepsins are synthesized as inactive preproenzymes and processed to active enzymes during maturation. Synthesis of cathepsins starts on membrane ribosomes with an amino-terminal signal peptide mediating the nascent polypeptide chain into the rough endoplasmic reticulum (for a review, see [3]). Following cotranslational cleavage of the presequence, the proenzymes are then transported to the Golgi complex for modification of the oligosaccharides to high-mannose carbohydrates and phosphorylation of the mannose residues. This modification allows the binding of procathepsins to membrane-bound mannose-6-phosphate receptors directing them into transport vesicles targeted to late endosomes (for reviews, see [4] and [5]). Within the acidic environment of late endosomes, the prodomain is cleaved converting the inactive zymogens into the mature active enzymes [6], [7]. Activation can occur either autocatalytically [8], [9], as exemplified, or through cleavage catalyzed by cathepsin D [6], [10], L or S [11] or other typical lysosomal proteases (for a review, see [5]).
Cathepsins are monomeric enzymes with molecular weights between 22 and 28 kDa with one exception; cathepsin C forms a tetrameric complex, which has a molecular weight of 200 kDa [12], [13]. They all have a papain-like fold revealed in the early days of crystallography [14]. However, structural characterization of cathepsins began in the early 1990s with the structure of cathepsin B [15]. Crystal structures of all human lysosomal cysteine cathepsins or their mammalian orthologs with the exception of cathepsins O and W are now available. In contrast to the structure of the processed form of cathepsins, only a few zymogen structures are available ([15–28]; for a review, see [29]). The papain-like fold consists of two domains, L and R, folded together displaying a V-shaped configuration. The active site lies at the interface between these two domains. The L-domain is mainly composed of three \( \alpha \)-helices whereas the R-domain is based on a \( \beta \)-barrel motif. The crystal structure of the zymogen already revealed the configuration of the activated mature enzyme. The prodomain forms an \( \alpha \)-helical domain and is folded in such a way that it blocks access to the active site cleft [30–33]. Propeptides are in fact inhibitors of their cognate enzymes, as shown for cathepsin K, L and S by kinetic analyses [34].

Substrates bind along the active site cleft of the mature enzyme in an extend conformation [35]. According to the Schechter and Berger [36] nomenclature, the subsites on the protease are called S (for subsites), and the substrate amino acid residues are called P (for peptide). The amino acid residues of the amino-terminal side of the scissile bond are named P1, P2, P3, P…, and those residues of the carboxy-terminal side are numbered P1’, P2’, P3’ P…’. By definition, the scissile bond is located between P1 and P1’ residues (Fig. 1).

**Figure 1.** Schematic depiction to illustrate the Schechter and Berger nomenclature.

Predominantly three substrate binding sites of cysteine cathepsins, S2, S1 and S1’, mediate main as well as side chain contacts between substrates and enzymes (for a review, see [37]). Whereas the S2 binding site is a deep pocket, *e.g.* in the case of cathepsins K, S, and L, the S1 and S1’ sites provide a binding surface [23], [25], [26], [31]. The S1’ pocket of cathepsins X and B is relatively deep and well-defined [28], [38] compared to the endopeptidases cathepsin K, S, and L. In addition, cathepsin B possesses a shallow S2’ subsite. The carboxydipeptidase
activity is attributed to the presence of an occluding loop, an extra peptide segment, which provides an appropriately spaced acceptor (His-110 and His-111) for the negatively charged C-terminal carboxylate of the substrate at the P2’ position [15]. Generally, interactions between the endopeptidases and the P3 and P2’ residues of the substrate are solely based on side chains. Thus, the papain-like cysteine proteases represent a special group of peptidases with a small number of substrate-binding sites compared to serine proteases and aspartic proteases.

Most of the enzymes are endopeptidases (cathepsins F, K, L, O, S, V, and W) in which the active-site cleft extends along the whole length of the two-domain interface. However, the papain-like cysteine peptidase subfamily also comprises exopeptidases (cathepsins B, C, H and X) (for reviews, see [29], [39]). Cathepsin B is a carboxydipeptidase, cathepsin C an aminodipeptidase, cathepsin H an aminomonopeptidase, and cathepsin X primarily possesses a carboxymonopeptidase activity. Among these exopeptidases, access to the substrate binding sites is restricted by additional features preventing the binding of longer peptidyl substrates and providing interaction with charged N or C chain termini of substrates; loops in the case of cathepsin B [15] and X [40–42], or propeptide parts in the case of cathepsin H [19] and C [17]. Beside their exopeptidase activity, cathepsins B and H also exhibit endopeptidase activity by conformational flexibility (for reviews, see [4], [39]).

Apart from determinants of gene expression, activity of papain-like cysteine cathepsins is regulated by a number of factors, with pH being the most important. The majority of cysteine proteases are unstable and weakly active at neutral pH and thus are optimized to function in acidic intracellular vesicles. Cathepsin precursors are inactive until they reach their appropriate localization, where activation generally requires an acidic environment, thus preventing indiscriminate activation following mislocalization (for reviews, see [33], [43]). Furthermore, the active site cysteine is readily oxidized preventing thiol-dependent proteolysis, therefore these enzymes need a reducing environment that is provided in the lysosomal compartments by a cysteine-specific lysosomal transport system [44]. Activity is also dependent on the balance between the amount of active enzyme and the amount of endogenous inhibitors. Numerous inhibitors of papain-like cysteine cathepsins have been described. The most abundant is the superfamily of cystatins, which bind tightly and essentially irreversibly to the corresponding cysteine proteases [45].

As soluble enzymes with a clear preference for slightly acidic pH conditions and reducing environments, cysteine cathepsin activity is mainly localized within the compartments of the endosomal-lysosomal system and traditionally believed to be responsible for bulk protein
turnover. By the use of cathepsin gene knockouts, functional redundancy of cathepsins regarding unspecific protein degradation was shown ([46–51]; for a review, see [52]). However, beside their role in non-selective protein turnover, gene knockouts also revealed specific and individual functions which are very important for normal cell processes (for a review, see [3]). These specific functions are often associated with restricted tissue localization of the cathepsins, as demonstrated for cathepsin S being predominantly expressed in spleen [53], the thymus and testis-specific cathepsin V [54] also known as cathepsin L2, and cathepsin K which is highly expressed in ovaries and osteoclasts [55]. Although cathepsins B, C, F, H, L, O, and X are ubiquitously or widely expressed (for a review, see [3]), this does not preclude them from being involved in more specialized processes because subcellular localization of cathepsins in different organelles and even at the cell surface under specific physiological circumstances must be considered ([56]; for a review, see [57]. An enormous amount of data describing the function of cathepsins has been achieved through the generation of gene-targeted knockout animal models (for a review, see [58]). A few remarks about the more specific functions of the five most extensively characterized cathepsins B, K, S, L and C are given in the following paragraphs.
1.2. FUNCTIONS OF CATHEPSINS B, K, S, L, AND C

Cathepsin B is involved in physiological processes, such as remodeling of the extracellular matrix, promoting cell migration during wound healing ([59]; for a review, see [60]), and apoptosis ([61], [62]; for reviews, see [63], [64]). In addition, cathepsin B-deficient mice show alterations in pancreas and thyroid physiology [65], [66], but do not display any severe phenotype suggesting that cathepsin B is not essential in normal development [65]. This is probably due to a functional redundancy of cathepsins B and L because combined deficiency in mice is lethal [67], which was not observed for single-deficient mice [51].

Cathepsin K was shown to be crucial for bone matrix degradation (for a review, see [43]). This enzyme was initially detected in osteoclasts, multinucleated cells mediating normal bone remodeling [55], [68], [69]. Later, cathepsin K mRNA was detected in a variety of tissues [70–72]. Cathepsin K is secreted by osteoclasts and degrades several components of the bone matrix including type I collagen, the main constituent of the organic matrix of bones, as well as osteopontin and osteonectin. The degradation of collagen mediated by cathepsin K occurs not only outside osteoclasts but also in lysosomes within the osteoclasts [73] because inhibition of cathepsin K by specific inhibitors or by cathepsin K antisense oligonucleotides results in the accumulation of undigested collagen fibrils in lysosomes within the osteoclasts (for a review, see [74]). Recently, beside its collagen degrading activity, a regulatory function in bone resorption was identified for cathepsin K [75].

Cathepsin S has been demonstrated to play an essential role in the MHC class II antigen presentation pathway. It is the major processing enzyme of the MHC class II invariant chain necessary for subsequent peptide loading ([49], [76–78]; for reviews, see [79], [80]), which was investigated by the use of a specific cathepsin S inhibitor [81]. Cathepsin S activity was quantified mainly in endosomes of antigen presenting cells using a novel specific substrate [82]. In addition, the repertoire of antigenic peptides in cathepsin S-deficient mice was changed providing an involvement of cathepsin S in the generation of antigenic peptides [78].

Also, cathepsin L (and F) seems to participate in processing of the MHC class II invariant chain, primarily in cells or tissues not expressing cathepsin S [47], [83]. Furthermore, cathepsin L is involved in epidermal homeostasis and regular hair-follicle morphogenesis and recycling; cathepsin L-deficient mice developed periodic hair loss and epidermal hyperplasia [51] similar to the phenotype of the furless mouse mutant [84]. Within secretory vesicles, cathepsin L is responsible for the generation of several peptide neurotransmitters and
hormones (for a review, see [85]). Recently, it was shown that cathepsin L participates in the
turnover of the extracellular matrix by conversion of proheparanase into its active form which
is able to degrade heparan sulfate in the extracellular matrix and on the cell
surface [86].

Cathepsin C (also referred to as dipeptidyl peptidase I) appears to be important for the
processing and activation of serine proteases in immune cells. For instance, cathepsin C is
involved in the conversion of pro-granzymes into proteolytically active enzymes. It was
reported that granzymes A and B from cytotoxic T-lymphocytes of cathepsin C-deficient mice
are mostly inactive ([50]; for a review, see [87]). Granzymes are serine proteases which are
required for cytotoxic lymphocyte granule-mediated apoptosis of target cells. Cathepsin C
also activates pro-inflammatory serine proteases, e.g. cathepsin G, neutrophil elastase and
proteinase-3, expressed in mature neutrophils involved in inflammation [88], [89].
1.3. INVOLVEMENT OF CATHEPSINS IN DISEASES

Imbalance of normal cathepsin activity is associated with a number of pathological events, including rheumatoid arthritis and osteoarthritis, inflammation, cancer, neurological disorders, multiple sclerosis, pancreatitis, diabetes, osteoporosis and lysosomal storage diseases. Cysteine cathepsins are important processing enzymes participating in proteolytic cascades, their altered expression and/or activity is involved in a high number of diseases (for reviews, see [43], [74], [90–99]). Furthermore, many of the diseases mentioned above correlate with subcellular mislocalization of these enzymes, for example outside lysosomes in the cytosol or in the extracellular environment (for a review, see [3]).

As mentioned before, cathepsin K is the major enzyme responsible for the degradation of the protein matrix of bone. The important role of cathepsin K for the function of osteoclasts was first suggested by the finding that cathepsin K activity deficiency induces pycnodysostosis, a disorder with an increase in bone mineral density (for reviews, see [29], [33], [43], [74]). This emerging evidence has made cathepsin K an important pharmacological target for the treatment of osteoporosis (for reviews, see [97], [98], [100]).

Cathepsin S has been discussed to be involved in autoimmune diseases such as rheumatoid arthritis because it mediates the degradation of the MHC class II invariant chain Ii, which is afterwards competent for binding antigenic peptides. Additionally, cathepsin S has been implicated in the degradation of endosomal/lysosomal proteins to generate the peptide fragments that are loaded into the MHC class II peptide-binding groove in human antigen-presenting cells ([49]; for reviews, see [43], [76–80]). Therefore, selective inhibition of cathepsin S is a potential mechanism for modulating the immune response. It has been shown that inhibition of cathepsin S alters autoantigen presentation and the development of organ specific autoimmunity in a murine model exhibiting an autoimmune disorder [101].

Cytosolic forms of cathepsin B, one of the most stable proteases at neutral pH, and cathepsins D and L, leaking out of the lysosomes, were shown to participate in apoptosis ([61], [62], [102]; for reviews, see [63], [64]). For instance, it was published recently that granulysin, a lipid-binding protein exhibiting antimicrobial activity, can target lysosomes of tumor cells and induce partial release of lysosomal contents into the cytosol, triggering programmed cell death [103]. Because a variety of different models confirm the biological importance of cathepsins in the regulation of apoptosis under physiological conditions, it is hence not surprising that altered activity or subcellular localization of cathepsins leads to
defects in the apoptotic mechanism closely related to the development of various diseases, in which the life span of cells is reduced (for a review, see [64]).

Several cysteine cathepsins were identified to be involved in cancer progression on the basis of their increased expression and/or activity in various human and mouse tumors ([104]; for reviews, see [91–96]). During cancer progression, cathepsins are often translocated to the cell surface or secreted into the extracellular milieu. They can still function outside tumor cells because the extracellular microenvironment of tumors is generally acidic (for a review, see [105]). In the extracellular milieu, cysteine cathepsins can promote tumor invasion either through uncontrolled cleavage of components of the extracellular matrix and basement membrane, through activation of growth factors or other proteases such as matrix metalloproteinases and urokinase plasminogen activator, which in turn promote invasion, or through cleavage of cell adhesion proteins (for a review, see [93]).

Several genetic disorders have been attributed to mutations in genes of cysteine cathepsins. For example, pycnodysostosis, characterized by severe bone abnormalities, is a rare, autosomal recessive skeletal disease caused by a mutation located within the cathepsin K gene [106]. On the other hand, loss-of-function mutation in the gene encoding cathepsin C leads do Papillon-Lefèvre syndrome, an autosomal recessive disorder, characterized by palmoplantar keratosis and severe early-onset periodontitis [107]. Similarly, disorders can also based on down-regulation of the endogenous inhibitors of cysteine cathepsins which is the case for the hereditary form of monoclonal epilepsy caused by a mutation in the gene encoding stefin B (cystatin B) ([108]; for reviews, see [17], [29]).

In addition, cathepsins were reported to be implicated in neurodegenerative diseases, such as Morbus Alzheimer. The formation of the amyloid β peptide (Aβ), deposited in the brain as amyloid plaques playing the major role in the pathogenesis of Alzheimer’s disease, is initiated by processing the amyloid precursor protein (APP) through cleavage by the two proteases β-secretase and γ-secretase. It is widely accepted that the major neuronal β-secretase is a transmembrane aspartyl protease termed BACE1 (β-site APP-cleaving enzyme 1) (for reviews, see [109], [110]). It has been proposed that cathepsin B may function as β-secretase [111]. However, an Aβ42 reducing activity of cathepsin B was demonstrated in a study with transgenic cathepsin B deficient mice, expressing human APP containing the Swedish and Indiana mutations at the β-secretase and γ-secretase sites. The authors proposed antiamyloidogenic and neuroprotective functions of cathepsin B [112]. On the other hand, it was reported that cysteine protease inhibitors reduced levels of brain Aβ40 and Aβ42 in a guinea pig model of human Aβ production [113]. Moreover, the β-secretase activity to cleave
the wild-type APP in regulated secretory vesicles, represented by cathepsin B, was suppressed by cysteine protease inhibitors. *In vivo* treatment of London APP mice with the inhibitors resulted in an improvement in memory deficit, a reduced amyloid plaque load and decreased Aβ40 and Aβ42 [114]. A recently published article showed that lowering Aβ levels in brain of cathepsin B-deficient mice depends on the type of APP expressed in these transgenic knockout mice. Whereas cathepsin B knockout in mice expressing human APP containing the rare Swedish and Indiana mutations had no effect on Aβ, the use of cathepsin B-deficient mice expressing human wild-type APP resulted in substantial decreases in brain Aβ. It was therefore concluded that cathepsin B is a target for inhibitors to lower Aβ in Alzheimer’s disease [115]. An epoxide inhibitor of cathepsin B as well as small interfering RNA produced decreases in Aβ42 release. It was discussed by the authors that cathepsin B acts as a secondary neuronal β-secretase, or indirectly modulates β-secretase activity [116]. Another cysteine cathepsin, cathepsin L, might also be involved in APP processing, even though this enzyme is responsible for the opposing effect, the reduction of Aβ42 levels [116].

It is now becoming clear that cysteine cathepsins should be seriously considered as potential drug targets in several diseases, most notably in a wide range of cancers, osteoporosis and autoimmune diseases. Small-molecule inhibitors directed against cathepsin K or cathepsin S are currently under clinical trials for the treatment of osteoporosis and bone metastasis, or for the treatment of autoimmune diseases ([137]; for reviews, see [74], [90], [92], [96], [100]).
1. INTRODUCTION

1.4. BONE REMODELING AND OSTEOPOROSIS

The human skeleton is metabolically active organ that undergoes continuous remodeling processes throughout the lifespan. Approximately 10% of human bone is replaced each year whereas the full renewal is completed every 10 years. The bone remodeling cycle involves a complex series of sequential steps with three main phases: (1) resorption of mineralized bone by osteoclasts; (2) appearance of mononuclear cells on the bone surface; and (3) formation of bone matrix by osteoblasts that subsequently become mineralized (Fig. 2) (for reviews, see [117], [118]).

![Figure 2. Bone remodeling cycle](image)

- (1) Resting bone surface; (2) Preosteoclasts; (3) Active osteoclasts; (4) Mononuclear cells; (5) Preosteoblasts; (6) Osteoblasts; (7) Osteocytes.

In turn, the osteoclastic bone resorption requires two processes: (1) demineralization of the inorganic bone components; and (2) degradation of the organic bone matrix. The first phase involves acid secretion by the osteoclast into the resorption lacunae, and the second phase is the proteolytic degradation of the organic bone matrix. The major proteolytic activity in osteoclasts is represented by cathepsin K with 98% of the total cysteine protease activity in these cells. Osteoclast-expressed cathepsin K is able to degrade several organic bone components including type I collagen, which constitutes approximately 90% of the organic bone matrix, as well as osteopontin and osteonectin (for reviews, see [97], [119]).

An imbalance between bone resorption and bone formation (also known as abnormal bone remodeling) can result in different diseases such as pycnodysostosis and osteoporosis. Osteoporosis is a skeletal disorder characterized by reduced bone density and micro-architectural deterioration leading to enhanced bone fragility and high risk for spontaneous fractures. Osteoporosis can be classified as primary type I (posmenopausal osteoporosis),
primary type II (senile osteoporosis) and secondary (with traceable aetiology) (for a review, see [120]). Aside from its human cost, osteoporosis is a considerable public health problem with enormous economic impact. For example, 3.79 million osteoporotic fractures were estimated in Europe in the year 2000 causing the total direct costs of 31.7 billion € [121].

Current treatment options of osteoporosis are mainly four classes of drugs: (1) calcitonins; (2) bone calcium regulators; (3) selective oestrogen receptor modulators (SERMs); and (4) parathyroid hormones (PTHs). Bisphosphonates, belonging to the bone calcium regulators, dominate the osteoporosis market as a gold standard for osteoporosis treatment. The most promising innovative antiosteoporotic drug is denosumab. Denosumab is a humanized monoclonal antibody that inhibits RANK-L (the ligand of receptor activator of nuclear factor-κB) preventing differentiation of osteoclast precursors into bone resorbing osteoclasts (for a review, see [122]). The Committee for Medicinal Products for Human Use (CHMP) adopted a positive opinion for denosumab on December 17, 2009. The approved indication for this drug is: (1) treatment of osteoporosis in postmenopausal women at increased risk of fractures; and (2) treatment of bone loss associated with hormone ablation in men with prostate cancer at increased risk of fractures [123]. A further interesting new drug candidate for osteoporosis treatment is odanacatib [13, Fig. 10]. Odanacatib is a potent and selective nitrile-containing cathepsin K inhibitor which is currently in the clinical development. Two studies have been carried out to evaluate the efficacy and safety of odanacatib. In a randomized, placebo controlled, double-blind phase I study in post-menopausal women, the optimal dose of odanacatib was determined. Reductions in resorption markers were greatest for doses > 50 mg weekly and doses ≥ 2.5 mg daily. In a further double-blind, randomized, placebo-controlled trial including 399 post-menopausal women, the efficacy and safety of odanacatib was evaluated. In this phase II study, statistically significant reductions of resorption markers were observed in patients receiving 50 mg odanacatib weekly. Furthermore, bone biopsies were carried out in 28 patients and showed no adverse histologic effects. To clarify the efficacy of odanacatib in term of fracture reductions, a phase III study is ongoing, with results expected in 2012. This is a clinical, randomized, double-blind trial with 16,000 patients [124].
1.5. ANTIGEN PRESENTATION AND AUTOIMMUNE DISEASES

Major histocompatibility complex class II molecules (MHC-II) are expressed by only few specialized antigen-presenting cell types including macrophages as well as dendritic and B cells. MHC-II molecules are involved in the presentation of antigenic fragments required in the endocytic pathway for the activation of CD4 positive T cells. MHC class II molecules are αβ heterodimers that are synthesized and assembled in the endoplasmic reticulum. The heterodimerisation is assisted by the invariant chain (Ii) chaperon molecule that additionally blocks the MHC-II peptide-binding groove preventing premature peptide loading. Furthermore, the invariant chain is important for the transport of assembled MHC class II molecules through the Golgi apparatus ([125]; for a review, see [126]). In generally, two main proteolytic events occur during MHC-II-mediated antigen presentation: (1) cleavage of the antigens to small antigenic peptides for the binding in the binding groove of MHC-II molecules; and (2) degradation of the invariant chain (Fig. 3).

![Figure 3. MHC-II-mediated antigen presentation and the role of cathepsin S [119].](image)

(1) MHC class II molecule (MHC-II); (2) Invariant chain (Ii); (3) CLIP; (4) Antigenic peptide; (5) Antigen.

Antigen processing seems to be a multienzyme-catalyzed process involving several cysteine and aspartic proteases. It was not possible until now to identify single proteases playing a crucial role in the cellular antigen degradation. In contrast, cathepsin S was described as the major processing enzyme of the invariant chain in the MHC-II-mediated antigen presentation confirmed by the delayed Ii processing in the cells isolated from cathepsin S deficient mice. Cathepsin S is capable to cleave the invariant chain leaving an approximately 15 amino acid
long class II associated invariant chain peptide (CLIP), which can be replaced by antigenic peptides (Fig. 3). It was further reported that the role of cathepsin S in the Ii degradation process in the cells from cathepsin S-deficient mice can be adopted by cathepsin F, which is expressed at high levels in macrophages, but not in dendritic and B cells. The proteolytic synergism of cathepsins S and F in the Ii degradation process could be used for the modulation of the immune response using selective inhibitors of these enzymes ([83]; for reviews, see [119], [126]).

Autoimmunity is understood as a failure of self tolerance. Because it is not possible to explain all autoimmune diseases by a single theory, it was suggested to consider the ‘mosaic’ of autoimmunity [127] involving genetic, hormonal, immunological, and environmental factors. Autoimmune diseases can be divided in systemic (e.g. systemic lupus erythematosus) and organ-specific (e.g. type I diabetes mellitus). It is further distinguished between diseases, in which the selection and regulation of T and B cells is generally altered, and diseases responsible for a particular antigen (for reviews, see [128], [129], [130]). The most important drugs currently used for the treatment of autoimmune diseases are immunosuppressive agents such as corticosteroids, cytotoxic compounds (azathioprine and 6-mercaptopurine), antimetabolits (methotrexate), and calcineurin inhibitors (cyclosporin A) (for a review, see [131]). The innovative therapeutic strategies for the treatment of autoimmune disorders includes follow therapeutic possibilities: (1) anti-CD4, anti-CD3 and anti-CD28 antibody treatment that could potentially be used to regulate T cell population; (2) lytic anti-CD20 therapy to regulate B cell population (rituximab for the treatment of rheumatoid arthritis and systemic lupus erythematosus [132]); and (3) therapies directed against cytocins important for the T cell growth (Il-2, Il-4, Il-7, Il-15, Il-21) and inflammation (TNF, Il-1, Il-6, IFN-γ). TNF monoclonal antibodies (infliximab [133]) and the TNF-receptor (TNFR) fusion protein (etanercept [134]) are already in clinical use (for a review, see [135]). Furthermore, cathepsin S is currently considered to be an interesting new target for the therapy of autoimmune disorders, since it was found that cathepsin S inhibitors prevents autoimmunity in murine models of Sjögren syndrome and myasthenia gravis [136], [137].
1.6. NITRILE-BASED INHIBITORS OF CYSTEINE CATHEPSINS

1.6.1. EARLY STUDIES

Inhibitors of cysteine cathepsins are mainly peptidic or peptidomimetic structures containing electrophilic groups prone to covalent interactions with the active-site cysteine. Among them, nitrile inhibitors receive the most attention in current drug discovery (for a review, see [178]). The first nitrile compounds, for which inhibition of a cysteine protease has been described, were acetamidoacetonitrile 1 and benzamidoacetonitrile 2 shown in Figure 4. These acylated glycine nitriles inhibited the plant protease papain at pH 7 with $K_i$ values of 31 mM and 390 µM, respectively [138]. Compound 4, N-acetyl-L-phenylalanyl-glycine-nitrile, represents a dipeptide nitrile with a decreased $K_i$ value (0.73 µM) towards papain [139], [140].

![Figure 4. Nitrile-based inhibitors of papain.](image)

The reaction of nitrile-based inhibitors with cysteine proteases involves the reversible formation of thioimidate adducts (Fig. 5) as it has been initially discovered for papain. This process can be considered as Pinner-type reaction. The Pinner reaction is an organic reaction of a nitrile with an alcohol under acid catalysis. The conversion results in an imidate which can be transformed to either an ester or an amidine. NMR experiments provided the first evidence for the formation of a covalent thioimidate link between nitrile inhibitors and papain, and also for the regeneration of the nitrile function when the adduct dissociates. It was reported in 1986 that the $^{13}$C-labeled dipeptide nitrile 5, upon interaction with papain, yielded a $^{13}$C NMR signal of the thioimidate at 182 ppm, which disappeared, when the
inhibitor was displaced from the active site cysteine [141]. In a similar study, a model thioimidate was prepared from 4 and N-acetyl-L-cysteine and used to assign the signal for the protein-bound thioimidate [142]. Complementary NMR results have also been reported with the \textsuperscript{13}C-labeled benzamidoacetonitrile 3 [143]. Compound 6, a dipeptide nitrile with L-Phe in P2 and DL-Leu in P1 position inhibited papain with $K_i = 5.8 \mu M$ [142].

![Figure 5. Interaction of cysteine proteases with nitrile-based inhibitors.](image)

A linear free energy correlation for binding of pairs of peptide aldehydes and peptide nitriles was found, indicating an overall similarity in the way in which these two groups of ligands bind to papain, regardless of the difference in geometry at the reacting carbon, \textit{i.e.} \textsuperscript{sp}$^{2}$ \textit{versus} sp for aldehyde \textit{versus} nitrile ligands; \textsuperscript{sp}$^{3}$ \textit{versus} \textsuperscript{sp}$^{2}$ for their adducts [144]. The carbon atom of the thioimidate adopts a trigonal geometry, different from that of tetrahedral hemithioacetals formed between cysteine proteases and aldehydes. Thus, the thioimidate structure resembles more closely the acyl-enzyme than the tetrahedral intermediate of the enzyme-catalyzed substrate hydrolysis (Fig. 6).

![Figure 6. The overall mechanism of cysteine protease-catalyzed substrate hydrolysis is considered to consist of a number of steps involving a covalent acyl-enzyme and transient anionic tetrahedral intermediates and transition states. The formation of the acyl-enzyme complex \textit{via} the tetrahedral intermediate is shown (top). The oxyanion is stabilized by interaction with hydrogen bond donors. Comparison of the geometries of hemithioacetal (left) and thioimidate (right) adducts with intermediates in the reaction pathway for substrate hydrolysis. Aldehyde inhibitors form hemithioacetals with the active site cysteine, nitrile inhibitors form thioimidates.](image)

The oxyanion hole of cysteine proteases contributes to catalysis by stabilizing inherently unstable transition states. This region in papain is defined by two hydrogen bond donors on
the backbone amide nitrogen of Cys25 and the side chain amide nitrogen of Gln19. Mutants of papain (Gln19Ala, Gln19Glu) have been used to determine the kinetic parameters for inhibition by Phe-Gly-derived aldehyde and nitrile inhibitors. Mutation of Gln19 caused an important loss of inhibition by the peptide nitrile inhibitor, and thioimidate adducts with the papain mutants were less stable. However, despite the structural similarity of the hemithioacetals to the tetrahedral intermediate, the affinity of the peptide aldehyde inhibitor was almost unaffected by Gln19 mutations [145].

As a further notable result of these early studies, it was found that amino acid or peptide-derived nitriles did show some selectivity for cysteine over serine proteases [140], [142], and the cyano group was not considered a typical ‘warhead’ for serine protease [146]. For nitrile inhibitors, the addition of the soft sulfur nucleophile of the cysteine proteases or the hard oxygen nucleophile of the serine proteases can be considered according to the principle of hard and soft acids and bases (HSAB), but has not been investigated so far [147], [148]. Noteworthy, the inhibition of the serine protease dipeptidyl peptidase IV (DPP-IV) by nitrile compounds has received much attention during the last years. Glucagon-like peptide 1 (GLP-1) receptor agonism has emerged as a validated approach for the treatment of type 2 diabetes [149]. Cyanopyrrolidines such as vildagliptin (7) and saxagliptin (8) are dipeptide nitriles with a basic amino group (Fig. 7). Vildagliptin and saxagliptin were characterized as reversible, slow-binding inhibitors of DPP-IV [146], [150]. The X-ray crystal structure of the DPP-IV: saxagliptin complex revealed the covalent attachment between the active site serine and the inhibitor nitrile carbon [151]. Thus, the so formed imidate corresponds to the thioimidate adduct of cysteine protease inhibition by nitriles.

**Figure 7.** Inhibitors of the serine protease DPP-IV for the treatment of type 2 diabetes.
1.6.2. CONVERSIONS OF THIOIMIDATE ADDUCTS

Chemical transformations of nitriles require strong acid/base conditions or the use of peroxides. Nitrile-hydrolyzing enzymes from microorganisms are known and the question raised whether cysteine proteases exhibit such an activity. In the case of papain [141], [142], [152], the thioimidate adduct was found to revert to nitrile, instead of hydrolyzing to form an amide (Fig. 8). However, a peptide nitrile hydratase activity was engineered by a single selected mutation Gln19Glu at the active site of papain. A $k_{cat}$ value, increased by a factor of at least $4 \times 10^5$, was observed at pH 5 for this papain variant compared to the wild type enzyme. The role of the glutamic acid residue is to participate in the acid-catalyzed hydrolysis of the thioimidate to the carboxamide by the provision of a proton. The more reactive protonated thioimidate readily undergoes nucleophilic attack by water [153]. Because of the well-known amidase activity of papain, the carboxamide is then transformed to the corresponding carboxylic acid (Fig. 8). The enzymatic hydrolysis of nitriles by papain mutant Gln19Glu was also studied in aqueous-organic media [154]. The proposed mechanism of nitrile hydratase activity of the papain mutant was supported by molecular dynamic studies [155].

![Figure 8. Mechanism for the nitrile hydratase (top) and amidase (bottom) activities of papain. The wild type enzyme displays very weak nitrile hydratase activity. The resulting carboxamide is hydrolyzed to the acid by the natural amidase activity of the enzyme.](image)

However, the papain-catalyzed conversion of benzamidoacetonitrile 2 or $N$-acyethyl-$L$-phenylalanyl-glycine-nitrile 4 to carboxylic acids via corresponding amides could be accomplished with high enzyme concentration in the presence of an external thiol as monitored with NMR and HPLC. An attack of $\beta$-mercaptoethanol or $N$-acetylcycteamine at
the thioimidate adduct produces a non-enzyme bound thioimidate, which is readily hydrolyzable. Once the amide is formed, papain catalyzes its conversion to the acid [156]. This pathway was later considered in an investigation with 9, a biaryl inhibitor of cathepsin K (Fig. 9). However, no detectable amounts of the corresponding amide 10 or acid 11 were observed in the HPLC profiles of the incubation mixture of 9, cathepsin K, and external thiols [157].

Figure 9. Structure of 9, a non-peptidic biaryl inhibitor of human cathepsin K, and its amide and acid analogues 10 and 11. Compound 9 (500 nM, \(i.e.\) 100-fold \(K_i\)) was a poor substrate for nitrilase activity of cathepsin K (enzyme concentration 1 \(\mu\)M, \(\beta\)-mercaptoethanol 1 mM, DTT 2.5 mM, pH 5.5, room temperature, 30 min). The amide 10 was readily converted to 11 by cathepsin K, and this amidase activity was blocked by the irreversible cysteine protease inhibitor E64.
1.6.3. SELECTIVE NITRILE-BASED INHIBITORS OF CATHEPSINS K AND S

Considerable scientific attempts have been made to develop highly potent and selective cathepsin K inhibitors, as it became obvious from a number of recent publications (for a review, see [178]). These efforts resulted in the discovery of balicatib (12) and odanacatib (13) for which several clinical trials have already been conducted (Fig. 10) [97], [124], [158]. The 1-amino-1-cyclohexanecarboxylic acid (homocycloleucine), contributing to the cathepsin K selectivity in balicatib (12), was discovered by the introduction of various substituents into the P2 position of Cbz-protected dipeptide nitriles. The following optimization of the P3 substituent led to the identification of balicatib as highly potent and selective cathepsin K inhibitor [159]. Unfortunately, a small number of patients during a phase II study in women with postmenopausal osteoporosis developed morphea-like skin changes under treatment with balicatib [160]. The lysosomal accumulation of balicatib due to its basic piperazine moiety was reported as a possible reason for the observed skin pathology [161]. Regarding this potential toxic effect, further clinical development of balicatib is on hold. In contrast, odanacatib is a non-basic selective cathepsin K inhibitor obtained by the combination of a relatively large biaryl substituent at the P3 position and a fluorinated P2 leucine moiety [162]. As it is shown in Figure 10, odanacatib (13) has a peptidomimetic structure in which the P3-P2 amide linker is replaced by a trifluoroethylamine group for the enhancement of the binding affinity [163]. Moreover, the unnatural cyclopropane moiety was introduced into the P1 position to stabilize the inhibitor molecule toward proteolytic degradation ([162]; for a review, see [164]). Odanacatib is currently in the phase III clinical development [124].

![Figure 10. Structures of the cathepsin K inhibitors balicatib (12) [159] and odanacatib (13) [162].](image-url)
unfavorable interactions with the S2 pocket of cathepsin K provided a rational for the selectivity of this inhibitor: IC$_{50}$ values of 0.4 nM (cathepsin S), 3058 nM (cathepsin K), 337 nM (cathepsin B), and 132 nM (cathepsin L) were reported for compound 14. Furthermore, the 2,6-dichlorobenzyl analogue 15 (IC$_{50}$ = 0.7 nM) was completely inactive against cathepsins K and B, while being 350-fold selective for cathepsin S over cathepsin L ([165]; for a review, see [178]). Furthermore, it was recently reported on the development of highly potent and selective cathepsin S inhibitors, containing electrophilic $\alpha$-ketoamide moiety (e.g. 16, Fig. 11) as a ‘warhead’, for which a patent application was carried out (for a patent evaluation, see [166]). These compounds bear gem-dialkyl substituted cycloaliphatic rings at the P2 position in combination with small aromatic or saturated heterocyclic/cycloaliphatic P3 substituents.

Figure 11. Structures of selective cathepsin S inhibitors 14–16.

Cathepsin S-selective inhibitors RWJ-445380 and CRA-028129 are already in the clinical development for rheumatoid arthritis and psoriasis, respectively. Unfortunately, neither the results of clinical trials nor the structures of compounds RWJ-445380 and CRA-028129 are available until now (for reviews, see [96], [167]).
1.6.4. AZADIPEPTIDE NITRILES AS INHIBITORS OF CYSTEINE PROTEASES

The isoelectronic replacement of the $\text{C}^\alpha\text{H}$ group by a nitrogen atom to give azapeptides is a common structural modification in the chemistry of peptides. Azapeptides have attracted much interest due to their unique properties and applications as peptidomimetics in a variety of biological systems. Recently, it was reported on proteolytically stable azadipeptide nitriles as a novel class of cysteine protease inhibitors with picomolar $K_i$ values towards therapeutically relevant cathepsins K, S and L. These azadipeptide nitriles were composed of a Cbz-protected P2 amino acid and a P1 aza-amino nitrile whose nitrogens are essentially alkylated for reasons of the synthetic access (Fig. 12) [168]. Furthermore, the azadipeptide nitriles proved to be strong inhibitors of falcipains 1 and 2 from *Plasmodium falciparum* parasite and showed further antimalarial activity [169].

![Figure 12. Cα/N-exchange leading to an azadipeptide nitrile.](image)

Azadipeptide nitriles exhibited dramatically improved activities toward tested cysteine proteases in contrast to the corresponding carba-analogues. The improved potency of azadipeptide nitriles can be explained by the formation of a resonance-stabilized isothiosemicarbazide adducts with the active-site cysteine of target enzymes (Fig. 13). Furthermore, it has been shown that the covalent isothiosemicarbazide adduct has a reversible nature ([168]; for a review, see [178]).

![Figure 13. Reversible formation of an isothiosemicarbazide adduct.](image)
In a recent publication, Yao and coworkers reported on the development of organelle-specific drug delivery systems to selectively transport azadipeptide nitriles into the lysosomes [170]. The reported delivery system consists of a Tat peptide derived from the human immunodeficiency virus transactivator protein covalently attached to a non-selective azadipeptide nitrile [168] using the ‘click’ chemistry (compound 17, Fig. 14). Biological testing showed that the Tat peptide was capable to promote delivery of the attached azadipeptide nitrile to the lysosomes of HepG2 cells.

**Figure 14.** Inhibitor-Tag conjugate L1-Tat.

Despite their excellent inhibitory activity, the reported azadipeptide nitriles were nonselective and it remained unclear whether the selectivity for a single target cathepsin can be achieved at all with this class of inhibitors.
1.7. ‘ACTIVITY-BASED’ PROBES

Chemical probes that bind into the catalytic site and allow for direct detection of the active enzyme form are termed ‘activity-based’ probes (ABPs), or ‘mechanism-based’ probes. ABPs consist mainly of three parts: (1) specific inhibitor; (2) linker; and (3) fluorescent or radioactive reporter. In contrast to chromogenic/fluorogenic substrates and reversible inhibitors, ABPs bind covalently to the active site in an irreversible manner. The applications for ABPs include de novo enzyme discovery, high-throughput screening, in vivo imaging, and diagnostic of certain diseases (for a review, see [171]).

Recently, it was reported on the development of specific fluorescent ABPs (e.g. 18, Fig. 15) that covalently label active caspases in vivo [172]. Caspases are proteolytic enzymes which belong to the C14D subfamily of cysteine proteases. Among them, caspase-3 has been identified as a key mediator of apoptosis in mammalian cells [173]. The monitoring of caspase-3 using specific ABPs allow therefore direct quantification of apoptosis to assess, e.g., chemotherapeutic response in cancer patients.

![Structure of the caspase probe AB50-Cy5 (18)](image)

**Figure 15.** Structure of the caspase probe AB50-Cy5 (18) [172].

Furthermore, a cell-permeable, radiiodinated, ‘activity-based’ probe [$^{125}$I]BIL-DMK (Fig. 16) was developed for the determination of cysteine cathepsin activity in whole-cell enzyme occupancy assays [174]. Non-selective activity-based probe [$^{125}$I]BIL-DMK contains a diazomethylketone moiety that covalently interacts as a ‘warhead’ with the active-site of cysteine cathepsins in an irreversible manner. [$^{125}$I]BIL-DMK is highly reactive toward the pharmaceutically important cathepsins L, S, K and B. Recently, it was reported that [$^{125}$I]BIL-
DMK was used to probe cathepsin S activity in the leukocyte fraction of the whole blood to study distribution and diurnal modulation of this enzyme [175].

![BIL-DMK (19)](image)

**Figure 16.** Structure of the radioiodinated cathepsin probe \([^{125}\text{I}]\text{BIL-DMK (19)}\) [175].

A further interesting activity based probe was developed for ultrasensitive *in situ* visualization of active glucocerebrosidase molecules. Deficiency of glucocerebrosidase causes a lysosomal storage disorder called Gaucher disease. For the diagnostic and treatment of Gaucher disease, it is very important to know the amounts of the active form of glucocerebrosidase in affected cells. The activity-based probe MDW941 (compound 20, Fig. 17) consist of an epoxide-containing cyclophellitol derivative covalently attached to a BODIPY fluorophore. Using MDW941, it was possible to determine the glucocerebrosidase activity by SDS-PAGE in cell lysates of normal individuals and in Gaucher material [176].

![MDW 941 (20)](image)

**Figure 17.** Structure of the glucocerebrosidase probe MDW941 (20) [176].

Although the development of fluorescent ‘activity-based’ probes for cathepsin X was recently published [177], no such ABPs are known for selective probing of cathepsins K and S until now. Therefore, the development of potent ‘activity-based’ probes for these enzymes, *e.g.* for diagnostic and monitoring of osteoporosis and autoimmune disorders, represents an interesting new topic in the field of Medicinal Chemistry.

Parts of the ‘Introduction’ of this work are congruent with the recently published review on nitrile inhibitors of cysteine cathepsins by Frizler *et al.* [178].
1.8. AIM

The aim of this work was the development of potent and selective nitrile-based peptidomimetic inhibitors for human cathepsins K and S, containing a proteolytically stable azadipeptide scaffold, as well as the preparation and characterization of ‘activity-based’ probes for these enzymes.

To obtain highly potent and selective cathepsin K inhibitors, the stepwise optimization of the inhibitor structure was planned (Fig. 18). In the phase I of the development process, various substituents had to be introduced into the P2 position of the Cbz-protected azadipeptide nitrile scaffold to obtain a lead structure for further optimization. In the phase II, the modification of the P3 moiety as well as of the P3-P2 linker was to be performed.

![Figure 18](image1.png)

**Figure 18.** An overview of the planned diversity points for the stepwise optimization of inhibitor molecules to achieve the selectivity for cathepsin K. \( R_1, R_2, R_3 \) = various substituents; \( *R_1, R_2^* \) = optimized substituents; \( R_4 = \text{Me or H; } X = \text{N, O or 0; } Y = \text{NMe or CH}_2, n = 0 \text{ or 1.} \)

In a second project of this work, the systematic scan for P3 substituents of the dipeptide nitrile scaffold, containing isobutylcysteinesulfone moiety [165] at the P2 position, had to be performed to explore the S3 binding pocket of cathepsin S (Fig. 19, left).

Finally, irreversible, fluorescent ‘activity-based’ probes for cathepsins K and S, bearing a vinyl sulfone ‘warhead’, were to be prepared and evaluated *in vitro* (Fig. 19, right).

![Figure 19](image2.png)

**Figure 19.** Systematic scan for P3 substituents (*left*) and fluorescent ‘activity-based’ probes (*right*).

\( R = \text{various substituents; } Y = \text{NMe or CH}_2; \ FG = \text{fluorescent group.} \)
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2.1. AZADIPEPTIDE NITRILES AS CATHEPSIN K INHIBITORS

2.1.1. SYSTEMATIC SCAN FOR P2 SUBSTITUENTS

To develop potent and selective nitrile inhibitors of cathepsin K, a systematic scan for P2 substituents of the azadipeptide nitrile scaffold was performed. For this purpose, various Cbz-protected L-amino acids 21–26 were converted into the corresponding mixed anhydrides [179–181] by the reaction with isobutyl chloroformate in the presence of N-methylmorpholine. The following addition of an aqueous solution of 1,2-dimethylhydrazine, which was previously displaced with sodium hydroxide from its dihydrochloride salt, provided the formation of 1,2-dimethylhydrazides 27–32. The crude products 27–32 were purified on silica gel by column chromatography and subjected to the final reaction with cyanogen bromide leading to the target compounds 33–38 (Scheme 1).


While 1,2-dimethylhydrazides 27–32, containing α-monosubstituted amino acids, were easily obtained in moderate yields, the reaction of Cbz-protected 1-amino-1-cyclohexanecarboxylic acid (homocycloleucine) 39 with 1,2-dimethylhydrazine was more complicated, although the same reaction conditions were used as in the case of 27–32. The 1,2-dimethylhydrazide 40 was formed in a very low yield, and it was further not possible to isolate the desired product from the obtained reaction mixture in a pure form. Therefore, the synthetic route to azadipeptide nitrile 42 had to be optimized. Different from the procedure noted in Scheme 1, compound 39 was activated via a mixed anhydride and reacted with 1,2-dimethylhydrazine dihydrochloride in the presence of triethylamine under water-free conditions. The new synthetic route was associated with a slight improved yield of compound 40 and allowed for isolation of this product in a pure form. Finally, the 1,2-dimethylhydrazide 40 was converted into the corresponding azadipeptide nitrile 42 by the reaction with cyanogen bromide.
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The carbazate 41 was isolated as the major byproduct resulting from the nucleophilic attack of 1,2-dimethylhydrazine at the carbonate carbon of the mixed anhydride (Scheme 2).

![Scheme 2. Synthesis of the homocycloleucine-derived azadipeptide nitrile 42.](image)

a) 1. ClCO$_2$Bu, TEA, THF, -25 °C; 2. (MeNH)$_2 \times$ 2HCl, THF, -25 °C to rt; b) BrCN, NaOAc, MeOH, rt.

For comparative investigations, the homocycloleucine-derived dipeptide nitrile 43 [159] was synthesized (Scheme 3). When compound 39 was reacted with aminoacetonitrile monosulfate in the presence of triethylamine, the dipeptide nitrile 43 was formed in a moderate yield of 53%. The carbamate 44 was identified as a main byproduct of the reaction.

![Scheme 3. Synthesis of the homocycloleucine-derived dipeptide nitrile 43.](image)

a) 1. ClCO$_2$Bu, TEA, THF, -25 °C; 2. H$_2$NCH$_2$CN $\times$ H$_2$SO$_4$, THF, -25 °C to rt.

Obviously, the presence of the gem-disubstituted carbon in the Chz-protected homocycloleucine-derived mixed anhydride was responsible for low yields of the desired coupling products. This effect was particularly strong when 1,2-dimethylhydrazine, a rather weak, but steric demanding nucleophile was used. For comparison, the mixed anhydride coupling of protected Cα-monosubstituted amino acids with aminoacetonitrile or 1,2-dimethylhydrazine occurred with less pronounced formation of carbamate or carbazate byproducts, respectively [168], [182]. Compounds 33–38 were synthesized by Friederike Lohr in the course of her diploma thesis under my supervision.
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2.1.2. KINETIC PLOTS (SLOW-BINDING INHIBITION) AND EQUATIONS

The inhibitory activity of compounds, interacting with their target enzymes in a competitive manner, is mainly characterized by the $K_i$ values. In contrast to IC$_{50}$ values, the true inhibition constant $K_i$ is independent from the substrate concentration and assay conditions and is therefore advantageous to evaluate competitive inhibitors. For example, $K_i$ values obtained under different assay conditions are directly comparable. The calculation of the $K_i$ value requires the knowledge of the Michaelis constant ($K_m$) defined as the substrate concentration for which the velocity of an enzyme reaction is half of its maximal value.

For the following kinetic investigations, the Michaelis constants were therefore determined. The target cathepsins L, S and B were assayed photospectrometrically in the presence of increasing concentrations of the chromogenic substrates Z-Phe-Arg-pNa, Z-Phe-Val-Arg-pNa and Z-Arg-Arg-pNa, respectively. The formation of $p$-nitroaniline was measured at 405 nm. Cathepsin K was assayed fluorometrically in the presence of the fluorogenic substrate Z-Leu-Arg-AMC. In the cathepsin K assay, the excitation wavelength was 360 nm and the corresponding wavelength for emission was 440 nm. The enzymes were assayed with at least seven different concentrations of the particular chromogenic or fluorogenic substrate in duplicate experiments. The steady-state reaction rates ($v_s$) were calculated as slopes of the progression lines by linear regression (Fig. 21a). To obtain the $K_m$ values, the steady-state velocities were further plotted against increasing substrate concentrations and analyzed by non-linear regression (Fig. 21b) using the Michaelis-Menten equation (Fig. 20a). The Lineweaver-Burk (Fig. 22a) and Hanes-Woolf (Fig. 22b) plots were performed to review possible systematic errors. The corresponding equations for Lineweaver-Burk and Hanes-Woolf plots are shown in Figure 20b and 20c, respectively.

\[
\begin{align*}
(a) \quad v_s &= \frac{V_{\text{max}} \times [S]}{K_m + [S]} \\
(b) \quad \frac{1}{v_s} &= \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \times \frac{1}{[S]} \\
(c) \quad \frac{[S]}{v_s} &= \frac{[S]}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}}
\end{align*}
\]

Figure 20. Michaelis-Menten (a), Lineweaver-Burk (b) and Hanes-Woolf (c) equations.

$v_s$ – steady-state velocity (M s$^{-1}$); $V_{\text{max}}$ – maximum reaction rate (M s$^{-1}$); [S] – substrate concentration (M); $K_m$ – Michaelis constant (M). In Figures 21 and 22, dimensionless units instead of concentrations are used at the ordinates.
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Figure 21. (a) Cathepsin K-catalyzed hydrolysis of Z-Leu-Arg-AMC (100 mM sodium citrate pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS, 2% DMSO, 50 µM DTT, 25 °C) (•) [S] = 30 µM; (•) [S] = 20 µM; (•) [S] = 10 µM; (•) [S] = 5 µM; (•) [S] = 3 µM; (•) [S] = 2 µM; (•) [S] = 1 µM; (•) [S] = 0.9 µM. (b) Plot of steady-state reaction rates (v_s) versus increasing concentration of Z-Leu-Arg-AMC. The non-linear regression of the data pairs (v_s, [S]) gave a $K_m$ value of 2.6 ± 0.3 µM.

Figure 22. (a) Lineweaver-Burk plot of $1/v_s$ versus $1/[S]$. $K_m = 3.0 ± 0.1$ µM. (b) Hanes-Woolf of $[S]/v_s$ versus $[S]$. Linear regression gave a $K_m$ value of 2.7 ± 0.1 µM.

The $K_m$ values and the corresponding standard errors, obtained by non-linear regression using the Michaelis-Menten equation (Fig 20a), are listed in Table 1. The final substrate concentrations in the inhibition assays were follows: (1) cathepsin L assay (human recombinant cathepsin L from Calbiochem) – 100 µM (= 10.0 $K_m$) Z-Phe-Arg-pNa; (2) cathepsin L assay (human isolated cathepsin L from Enzo Life Sciences) – 100 µM.
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(= 5.88 $K_m$) Z-Phe-Arg-pNa; (3) cathepsin S assay (human recombinant cathepsin S from Calbiochem) – 100 µM (= 1.49 $K_m$) Z-Phe-Val-Arg-pNa or 100 µM (= 0.85 $K_m$) Z-Phe-Arg-pNa; (4) cathepsin K assay (human recombinant procathepsin K from Calbiochem) – 40 µM (= 15.4 $K_m$) Z-Leu-Arg-AMC; (5) cathepsin K assay (recombinant cathepsin K from Enzo Life Sciences) – 40 µM (= 13.3 $K_m$) Z-Leu-Arg-AMC; (6) cathepsin B assay (human isolated cathepsin B from Calbiochem) – 500 µM (= 0.45 $K_m$) Z-Arg-Arg-pNa.

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<th>Table 1. $K_m$ values (µM).</th>
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$^a$Assays were carried out with a total DMSO amount of 2% and at 37 °C. $^b$Assay was performed with a total DMSO amount of 2% and at 25 °C. $^c$Human recombinant cathepsin L from Calbiochem. $^d$Human isolated cathepsin L from Enzo. $^e$Not determined. $^f$Human recombinant procathepsin K from Calbiochem (measurements were performed after activation of the enzyme). $^g$Human recombinant cathepsin K from Enzo.

The inhibitory activities of the target compounds 33–38 and 42, together with already known inhibitors 45 and 46 [168] (Table 2), were evaluated on therapeutically relevant human cathepsins L, S, K and B as described in 'Experimental Section'. The tested azadipeptide nitriles all showed slow-binding kinetic behavior resulting in hyperbolic progress curves of enzymatic reactions in the presence of inhibitor (Fig. 24a). For calculation of the steady-state reaction rates ($v_s$) and the first-order rate constants ($k_{obs}$), the slow-binding equation was used (Fig. 23a). To obtain the IC$_{50}$ values (apparent inhibition constants $K'_i$), the steady-state velocities ($v_s$) were plotted versus increasing inhibitor concentrations (Fig. 24b), and the resulting data pairs ($v_s$, [I]) were analyzed by non-linear regression using the IC$_{50}$ equation (Fig. 23b). Because azadipeptide nitriles were described as reversible inhibitors with a competitive inhibition mode [168], the IC$_{50}$ values were further corrected using the Cheng-Prusoff equation (Fig. 23c) to obtain the corresponding true inhibition constants ($K_i$). The structure-activity relationships (SARs) of all inhibitors in this study were analyzed on the bases of their $K_i$ values because the true inhibition constants are independent on substrate concentrations and used assay conditions, and allow therefore for a direct comparison.
2. RESULTS AND DISCUSSION

(a) \[ E/I = v_s t + \left( \frac{v_i - v_s}{k_{obs}} \right) \times (1 - e^{-k_{obs}t}) + d \]

(b) \[ \text{IC}_{50} = \left( \frac{v_0}{v_s} - 1 \right) \]

(c) \[ K_i = \frac{\text{IC}_{50}}{1 + \frac{[S]}{K_m}} \]

Figure 23. Slow-binding (a), IC\(_{50}\) (b) and Cheng-Prusoff (c) equations.

(a) \(E\) – extinction; \(I\) – fluorescence intensity; \(v_s\) – steady-state reaction rate (M s\(^{-1}\)); \(v_i\) – initial reaction rate (M s\(^{-1}\)); \(k_{obs}\) – first-order rate constant (s\(^{-1}\)); \(t\) – time (s); \(d\) – offset; (b) IC\(_{50}\) – half maximal inhibitory concentration (M); \(v_0\) – steady-state reaction rate in the absence of inhibitor (M s\(^{-1}\)); \(v_s\) – steady-state reaction rate in the presence of inhibitor (M s\(^{-1}\)); \([I]\) – inhibitor concentration (M); (c) \(K_i\) – true inhibition constant (M); IC\(_{50}\) – half maximal inhibitory concentration (M); \([S]\) – substrate concentration (M); \(K_m\) – Michaelis constant (M). In Figure 24, dimensionless units instead of concentrations are used at the ordinates.

Figure 24. (a) Monitoring of the human cathepsin L-catalyzed hydrolysis of Z-Phe-Arg-pNa (100 µM) in the presence of increasing concentrations of compound 35 (•, 0; •, 20 nM; •, 40 nM; •, 60 nM; •, 80 nM; •, 100 nM). The reaction (100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01% Brij 35, 2% DMSO, 100 µM DTT, 37 °C) was initiated by addition of the enzyme. The formation of \(p\)-nitroaniline was measured at 405 nm. (b) Plot of the rates of hydrolysis of Z-Phe-Arg-pNa versus increasing concentrations of 35. Non-linear regression gave an apparent inhibition constant \(K_i' = (1+[S]/K_m)K_i = 17 \pm 1\) nM. The inset is a plot of the first-order rate constants \(k_{obs}\) versus increasing concentrations of 35. Linear regression gave a second-order rate constant \(k_{on}' = k_{on}(1+(S)/K_m) = (19 \pm 1) \times 10^3\) M\(^{-1}\)s\(^{-1}\).
The slow-binding behavior of azadipeptide nitriles, which inhibited the target cathepsins in a time-dependent manner, approves for the determination of the association rate constants ($k_{on}$) as a second kinetic parameter to evaluate their biological activity. Slow-binding inhibitors display a slow onset of the inhibition on a time scale of seconds to minutes because the equilibrium between enzyme, inhibitor, and enzyme-inhibitor (EI) complex is established slowly. Two basic mechanisms have been proposed to explain slow enzyme inhibition in the case of reversible, covalent inhibitors. Mechanism A assumes the formation of the covalent complex EI* as a single, slow step whereby the magnitudes of $k_{+3}[I]$ and $k_{-3}$ are small relative to $k_{+1}[S]$, $k_{-1}$ and $k_{+2}$ (Fig. 25a). However, mechanism B assumes rapid formation of a non-covalent EI complex that undergoes a slow isomerization to a covalent EI* complex. In this case, the magnitudes of $k_{+4}$ and $k_{-4}$ are small relative to $k_{+1}[S]$, $k_{-1}$ and $k_{+2}$, and in particular to $k_{+3}[I]$ and $k_{-3}$ (Fig. 25b) [183], [184].

(a)       (b)

![Figure 25. Mechanism A (a) and mechanism B (b) of the slow-binding inhibition.](image)

For the differentiation between two mechanisms, the $k_{obs}$ values (first-order rate constant for the development of the steady-state between free enzyme, inhibitor, and EI* complex), obtained from hyperbolic progress curves, were plotted against increasing inhibitor concentrations. As it exemplarily depicted for compound 35 (Fig. 24b), the $k_{obs}$ values of azadipeptide nitriles 33–38, 42, 45 and 46 showed a linear dependence on the increasing inhibitor concentrations. The linear dependence of $k_{obs}$ on [I], which is mathematically described by equation shown in Figure 26a, indicated the one-step mechanism A (Fig. 25a) as the inhibition mode of compounds 33–38, 42, 45 and 46 on cathepsins L, S, K and B. The apparent $k_{+3}(1+[S]/K_m) = k_{on}$’ values were therefore easily obtained by linear regression of the data pairs ($k_{obs}$, [I]) using the equation depicted in Figure 26c. In the case of mechanism B, the $k_{obs}$ values exhibit non-linear dependency on [I] described by the equation for two step inhibition (Fig. 26b).
2. RESULTS AND DISCUSSION

(a) \[ k_{\text{obs}} = \frac{k_{+3} \times [I]}{1 + \frac{[S]}{K_m}} + k_{-3} \]

(b) \[ k_{\text{obs}} = \frac{k_{+4} \times [I]}{k_{3} \times \left(1 + \frac{[S]}{K_m}\right) + [I]} + k_{-4} \]

(c) \[ k_{\text{obs}} = k_{\text{on}} \times [I] + k_{\text{off}} \]

Figure 26. Relationships between \(k_{\text{obs}}\) and \([I]\) for mechanism A (a) and mechanism B (b); equation for the determination of the apparent second-order association rate constants (c).

\(k_{\text{obs}}\) – first-order rate constant of the approach to steady-state (s\(^{-1}\)); \(k_{-3}\) – dissociation first-order rate constant (s\(^{-1}\)); \(k_{+3}\) – association second-order rate constant (M\(^{-1}\)s\(^{-1}\)); \(k_{3}k_{+3}\) – dissociation constant (M); \(k_{+4}\) – first-order rate constant of the EI* decay (s\(^{-1}\)); \(k_{-4}\) – first-order rate constant of the EI* formation (s\(^{-1}\)); \([I]\) – inhibitor concentration (M); \([S]\) – substrate concentration (M); \(K_m\) – Michaelis constant (M); \(k_{\text{on}}\) – apparent second-order association rate constant (M\(^{-1}\)s\(^{-1}\)); \(k_{\text{off}}\) – first-order dissociation rate constant (s\(^{-1}\)).

The calculated apparent second-order association rate constants \((k_{\text{on}}')\) were further corrected by multiplication with a factor \((1+\frac{[S]}{K_m})\) to obtain the corresponding true second-order association rate constants \((k_{\text{on}})\). The first-order dissociation rate constants \((k_{\text{off}})\) of compounds 33–38, 42, 45 and 46 were calculated as a multiplication product of \(K_i\) and \(k_{\text{on}}\) using the equation in Figure 27a. The standard errors for \(k_{\text{off}}\) were obtained by multiplication of \(k_{\text{off}}\) values with their relative overall errors (Fig. 27b) [185–187].

(a) \[ K_i = \frac{k_{\text{off}}}{k_{\text{on}}} \]  

(b) \[ F_{\text{rel}} = \sqrt{(f_{\text{rel}})^2 + (f_{\text{rel}})^2} \]

Figure 27. Equations for calculation of \(k_{\text{off}}\) values (a) and their relative overall errors (b).

(a) \(k_{\text{obs}}\) – first-order rate constant of the steady-state development (s\(^{-1}\)); \(k_{\text{on}}\) – true second-order association rate constant (M\(^{-1}\)s\(^{-1}\)); \(k_{\text{off}}\) – first-order dissociation rate constant (s\(^{-1}\)); (b) \(F_{\text{rel}}\) – relative overall standard error; \(f_{\text{rel}}\) – relative standard errors for \(K_i\) and \(k_{\text{on}}\) values.
2.1.3. STRUCTURE-ACTIVITY RELATIONSHIPS OF 33–38, 42, 45 AND 46

Regardless of the substitution pattern, compounds 33–38, 42, 45 and 46 displayed all the time-dependent, slow-binding behavior. Analysis of the progress curves over 80 min then allows for the determination of the second-order association rate constants ($k_{on}$) and the first-order dissociation rate constants ($k_{off}$). The estimated $K_i$ values of compounds 33–38, 42, 45 and 46 as well as the corresponding standard errors are shown in Table 2.

**Table 2. $K_i$ values of carbamate-based compounds 33–38, 42, 45 and 46.**

<table>
<thead>
<tr>
<th>cmpd</th>
<th>R</th>
<th>R’</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cathepsin L</td>
</tr>
<tr>
<td>33</td>
<td>H</td>
<td>H</td>
<td>860 ± 60$^a$</td>
</tr>
<tr>
<td>34</td>
<td>Me</td>
<td>H</td>
<td>480 ± 50</td>
</tr>
<tr>
<td>35</td>
<td>i-Pr</td>
<td>H</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>36</td>
<td>(S)-1-methylpropyl</td>
<td>H</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>37</td>
<td>cyclohexylmethyl</td>
<td>H</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>38</td>
<td>4-hydroxybenzyl</td>
<td>H</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>42</td>
<td>-(CH$_2$)$_5$-</td>
<td>H</td>
<td>400 ± 60</td>
</tr>
<tr>
<td>45</td>
<td>i-Bu</td>
<td>H</td>
<td>0.90 ± 0.04</td>
</tr>
<tr>
<td>46</td>
<td>Bn</td>
<td>H</td>
<td>0.16 ± 0.01$^a$</td>
</tr>
</tbody>
</table>

$^a$The progress curves were followed over 20 min and analyzed by non-linear regression.

The glycine derivative 33 with unsubstituted P2 position exhibited the weakest inhibitory activity on the studied cathepsins. Furthermore, the simple methylation of the P2 position, resulting in compound 34, led to a ca. 2–88-fold improvement in the potency towards cathepsins L, S, K and B. The activity improvement was much stronger when larger aliphatic and cycloaliphatic groups such as isopropyl (35), (S)-1-methylpropyl (36), isobutyl (45), and cyclohexylmethyl (37) substituents were introduced in the P2 position of the azadipeptide nitrile scaffold. Particularly for cathepsin K, the L-leucine-based azadipeptide nitrile 45 was
ca. 4100-fold more active, comparing with the corresponding glycine derivative 33, and showed also a slight preference for this enzyme. The introduction of aromatic benzyl (46) and 4-hydroxybenzyl (38) substituents into P2 position did not result in further advantages regarding potency improvement or selective inhibition of cathepsin K. In contrast, the homocycloleucine-based compound 42, although less potent on cathepsin K compared with the potent inhibitors 45 and 37, showed already considerable selectivity for this enzyme over cathepsin L, S and B (220-fold, 72-fold and 100-fold, respectively). Azadipeptide nitriles 42 and 45 were selected for further optimization of the P3 substituent, while their P2 residues, interacting with the S2 subsite of cysteine cathepsins, were maintained in all further compounds of this study.

The $k_{on}$ values of compounds 33–38, 42, 45 and 46 varied considerably depending on the substitution at the P2 position, while the corresponding $k_{off}$ values were in the same range (Tables 3 and 4). For example, the 17000-fold higher $k_{on}$ value of 45 for cathepsin K, compared to that of the glycine derivative 33, is reflected by the improved potency of 45 (64 pM versus 260 nM, Table 2).

### Table 3. $k_{on}$ values of carbamate-based compounds 33–38, 42, 45 and 46.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>R</th>
<th>R'</th>
<th>$k_{on}$ (10^3 \text{M}^{-1} \text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>33</td>
<td>H</td>
<td>H</td>
<td>0.51 ± 0.01$^a$</td>
</tr>
<tr>
<td>34</td>
<td>Me</td>
<td>H</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>35</td>
<td>i-Pr</td>
<td>H</td>
<td>210 ± 10</td>
</tr>
<tr>
<td>36</td>
<td>(S)-1-methylpropyl</td>
<td>H</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>37</td>
<td>cyclohexylmethyl</td>
<td>H</td>
<td>2900 ± 100</td>
</tr>
<tr>
<td>38</td>
<td>4-hydroxybenzyl</td>
<td>H</td>
<td>4200 ± 100</td>
</tr>
<tr>
<td>42</td>
<td>-(CH$_2$)$_5$-</td>
<td></td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>45</td>
<td>i-Bu</td>
<td>H</td>
<td>620 ± 50</td>
</tr>
<tr>
<td>46</td>
<td>Bn</td>
<td>H</td>
<td>4500 ± 700$^a$</td>
</tr>
</tbody>
</table>

$^a$The progress curves were followed over 20 min and analyzed by non-linear regression.
The $k_{\text{off}}$ values of tested compounds 33–38, 42, 45 and 46 were approximately in the same range and seemed to be independent from the substituent at the P2 position of the azadipeptide nitrile scaffold (Table 4). Therefore, it could be assumed that azadipeptide nitriles depart from the catalytic site of cysteine cathepsins without significant interactions with the corresponding binding pockets when the dissociation of the isothiosemicarbazide (Fig. 13) adducts occurs. This finding reflects the difficulty in delivering significant binding energy from non-covalent interactions, as discussed for cysteine cathepsins and concluded from the large and relatively shallow active site of these target enzymes [164]. The cathepsin L measurements of compounds 33–38 were performed by Friederike Lohr in the course of her diploma thesis under my supervision.

### Table 4. $k_{\text{off}}$ values of carbamate-based compounds 33–38, 42, 45 and 46.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>R</th>
<th>R'</th>
<th>$k_{\text{off}} \times 10^{-3}$</th>
<th>cath L</th>
<th>cath S</th>
<th>cath K</th>
<th>cath B</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>H</td>
<td>H</td>
<td>0.44 ± 0.03$^a$</td>
<td>1.4 ± 0.1</td>
<td>0.026 ± 0.004</td>
<td>0.092 ± 0.018</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Me</td>
<td>H</td>
<td>0.62 ± 0.08</td>
<td>0.61 ± 0.07</td>
<td>0.046 ± 0.007</td>
<td>0.045 ± 0.018</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>i-Pr</td>
<td>H</td>
<td>0.32 ± 0.03</td>
<td>0.60 ± 0.17</td>
<td>0.070 ± 0.014</td>
<td>0.033 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>(S)-1-methylpropyl</td>
<td>H</td>
<td>0.21 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.074 ± 0.017</td>
<td>0.025 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>cyclohexylmethyl</td>
<td>H</td>
<td>1.2 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>0.029 ± 0.005</td>
<td>0.028 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>4-hydroxybenzyl</td>
<td>H</td>
<td>1.5 ± 0.1</td>
<td>0.43 ± 0.03</td>
<td>0.051 ± 0.005</td>
<td>0.030 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>-(CH$_2$)$_5$-</td>
<td>H</td>
<td>0.52 ± 0.09</td>
<td>1.0 ± 0.1</td>
<td>0.11 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>i-Bu</td>
<td>H</td>
<td>0.56 ± 0.05</td>
<td>0.66 ± 0.05</td>
<td>0.11 ± 0.01</td>
<td>0.056 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Bn</td>
<td>H</td>
<td>0.72 ± 0.12$^a$</td>
<td>1.5 ± 0.3$^a$</td>
<td>0.045 ± 0.009</td>
<td>0.10 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The progress curves were followed over 20 min and analyzed by non-linear regression.
2. RESULTS AND DISCUSSION

2.1.4. L-LEUCINE-DERIVED AZADIPEPTIDE NITRILES

The L-leucine-based azadipeptide nitrile 45 was selected for the stepwise structure optimization to develop potent and selective cathepsin K inhibitors. For this purpose, the isobutyl substituent at the P2 position of the azadipeptide nitrile scaffold was maintained, and the P3 moieties as well as the P3-P2 linker were varied.

At first, the carbamate group of 45 was replaced by a urea moiety to permit additional hydrogen bond formation. The corresponding azadipeptide nitriles 50 and 51 (Scheme 4) differ in the length of the P3-P2 linker. The route to the urea-based inhibitors 50 and 51 rests upon the finding that the carbamoyl-protected L-leucine can be converted via mixed anhydride method without racemisation [188]. L-Leucine tert-butyl ester 47 was reacted with CDI and benzylamine or aniline, respectively. The resulting derivatives 48 and 49 were treated with TFA to cleave the tert-butyl ester, transformed into the corresponding 1,2-dimethylhydrazides, and reacted with cyanogen bromide to obtain 50 and 51.

Scheme 4. Synthesis of the urea-based compounds 50 and 51.

On the basis of recent results on cathepsin K-selective dipeptide nitriles with large biaryl P3 substituents [164], the P3 moiety of compounds 50 and 51 was next extended to improve the potency and selectivity for cathepsin K over cathepsins L, S and B. A 1,2,4-oxadiazole heterocycle as a common bioisoster of amide and ester groups [189] was chosen for the extension. Two benzylurea derivatives bearing a methyl group (62) and a 2-thienyl substituent (63) in position 5 of the 1,2,4-oxadiazole ring, as well as their phenylurea counterparts 64 and 65 were synthesized (Scheme 5). The synthetic route includes the conversion of aromatic nitriles 52 and 53 to acyloxyamidines 54–57, and the oxadiazole ring closure carried out in acetic acid at 80 °C [190], followed by deprotection with TFA to obtain compounds 58–61. The free acids 58–61 were activated via a mixed anhydride and reacted with 1,2-dimethyl-
hydrazine to obtain the corresponding 1,2-dimethylhydrazides, which were finally converted to azadipeptide nitriles 62–65 with cyanogen bromide.


For a direct comparison of kinetic properties, compound 66 was further synthesized as a carba-analogue of the azadipeptide nitrile 65. The synthetic route to 66 includes the activation of the free acid 61 via a mixed anhydride and the following reaction with aminoacetonitrile monosulfate (Scheme 6).


As it became obvious with inhibitor 65, the combination of the extended P3 triaryl motif with a short P3-P2 linker was advantageous, it was decided to further reduce the linker leading to the design of amide-based azadipeptide nitriles 67 and 68 (Scheme 11). Because of the racemisation of N-acylamino acids known to occur during the activation of their
carboxylic groups, a convergent synthesis for the amide-based compounds was developed. To realize the planned synthetic route, the desired compounds 67 and 68 were retrosynthetically divided into the synthetic equivalents 69, 70, 71 and cyanogens bromide (Fig. 28) which should be assembled to target molecules. While cyanogen bromide was commercially available, the building blocks 69 and 70, 71 had to be prepared.

Figure 28. The planned synthetic route to compounds 67 and 68.
67, 70, R = H; 68, 71, R = Me.

The convergent synthetic route was first employed in a modified form for the carba-analogues 79 and 80 (Scheme 8). At first, the P3 building block 69 was prepared in five steps with an overall yield of 49% (Scheme 7). The crucial step to compound 69 was the formation of the acyloxyamidine 74, followed by the formation of 1,3,4-oxadiazole ring and the ester cleavage.

Scheme 7. Synthesis of compound 69 as the P3 building block.
a) 1. (COCl)$_2$, CH$_2$Cl$_2$, DMF, rt; 2. tert-butanol, pyridine, rt; b) NH$_2$OH × HCl, DIPEA, EtOH, Δ; c) 2-thenoyl chloride, DIPEA, MeCN, rt; d) AcOH, 80 ºC; e) TFA, CH$_2$Cl$_2$, rt.
Next, Boc-protected L-leucine 75 and N-methyl-L-leucine 76 were reacted with aminoacetonitrile, followed by removal of the protecting group using methanesulfonic acid and basic extraction to produce 77 and 78. In the final step, the building block 69 was activated and coupled with 77 and 78 to obtain dipeptide nitriles 79 and 80 (Scheme 8).

**Scheme 8.** Synthesis of amide-based dipeptide nitriles 79 and 80.

a) 1. ClCO₂-i-Bu, NMM, THF, -25 °C, 2. H₂NCH₂CN × H₂SO₄, NaOH, H₂O, THF, -25 °C to rt; b) MeSO₃H, THF, rt, basic extraction; c) (R = H) 69, ClCO₂-i-Bu, NMM, THF, -25 °C to rt; (R = Me) 69, EDC, DMAP, CH₂Cl₂, rt; 75, 77, 79, R = H; 76, 78, 80, R = Me.

To produce the synthetic equivalents 70 and 71 (Fig. 28) for the synthesis of the desired amide-based azadipeptide nitriles 67 and 68, it was intended to first protect the terminal hydrazide nitrogen of 82, followed by the deprotection of the α-amino group. However, after treatment of 82 with (Boc)₂O in acetonitrile and in the presence of DMAP, the 1,2,4-triazinane-3,6-dione 84 was obtained, instead of the orthogonal protected azadipeptide ester 83 (Scheme 9). The attack of the NH nitrogen at the carbazate carbon operates in this ring closure. Although the sodium hydride-promoted formation of the 1,2,4-triazinane-3,6-dione scaffold has been described [191], the reaction to compound 84 represents the first example of a direct 1,2,4-triazinane-3,6-dione cyclization of a Cbz-protected azadipeptide tert-butyl ester.

**Scheme 9.** Formation of 1,2,4-triazinane-3,6-dione derivative 84.

a) 1. ClCO₂-i-Bu, NMM, THF, -25 °C, 2. (MeNH)₂ × 2HCl, NaOH, H₂O, THF, -25 °C to rt; b) (Boc)₂O, DMAP, MeCN, rt.
The cyclisation reaction of Cbz-protected 1,2-dimethylhydrazides to 1,2,4-triazinane-3,6-diones by the reaction with (Boc)_2O in the presence of DMAP, as described in this work, represents an easy synthetic access to new potential peptidomimetic structures with at least four diversity points (Scheme 10).

Scheme 10. Synthetic access to potential peptidomimetic 1,2,4-triazinane-3,6-diones.
(Boc)_2O, DMAP, MeCN, rt.

The finally successful synthesis of amide-based dipeptide nitriles 67 and 68, the aza-analogues of 79 and 80, is outlined in Scheme 11. The Cbz protecting group of the 1,2-dimethylhydrazides 82 and 86 was hydrogenolytically removed to obtain derivatives 87 and 88. The synthetic equivalent 69 (Fig. 28) was activated with EDC in the presence of DMAP and reacted with 87 and 88 leading to the formation of compounds 89 and 90. In the final reaction step with cyanogen bromide, the amide-based azadipeptide nitriles 67 and 68, bearing a large P3 substituent, were obtained.

Scheme 11. Synthesis of amide-based azadipeptide nitriles 67 and 68.
a) 1. ClCO_2i-Bu, NMM, THF, -25 °C, 2. (MeNH)_2 × 2HCl, NaOH, H_2O, THF, -25 °C to rt; b) H_2, Pd/C, 2 bar, MeOH, rt; c) 69, EDC, DMAP, CH_2Cl_2, rt; d) BrCN, NaOAc, MeOH, rt; 81, 82, 87, 89, 67, R = H; 85, 86, 88, 90, 68, R = Me.
2. RESULTS AND DISCUSSION

2.1.5. KINETIC PLOTS II (FAST-BINDING INHIBITION)

Urea-based and amide-based azadipeptide nitriles were tested on human cathepsins L, S, K and B as described in ‘Experimental Section’. Azadipeptide nitriles 50, 51, 62–65, 67 and 68 showed a time-depended slow-binding inhibition type similar to their carbamate-derived counterparts, while the carba-analogues 66, 79 and 80 appeared as fast-binding inhibitors.

The kinetic parameters $K_i$, $k_{on}$, and $k_{off}$ of slow-binding inhibitors 50, 51, 62–65, 67 and 68 were calculated as described in chapter 2.1.2. Before the IC$_{50}$ and $k_{on}$ were corrected to zero-substrate concentration, the reversibility of the isothiosemicarbazide formation (Fig. 13) was shown in a reactivation experiment with compound 68 [192].

In the case of fast-binding inhibitors 66, 79 and 80, the progress lines were analyzed by linear regression (Fig. 29a), and the resulting steady-state rates ($v_s$) were plotted against [I]. The corresponding IC$_{50}$ values were obtained by non-linear regression of the data pairs ($v_s$, [I]) as shown in Figure 29b. The IC$_{50}$ values were corrected using the Cheng-Prusoff equation (Fig. 23c) to obtain the true inhibition constants ($K_i$).

![Figure 29](image)

In general, the replacement of the carbamate P3-P2 linker by a urea moiety resulted in an improved inhibitory activity of urea-based azadipeptide nitriles toward cathepsins L, S, K and B. With $K_i$ values of 11 and 17 pM, respectively, compounds 50 and 51 were even more potent for cathepsin K than the corresponding carbamate-based derivative 45 (Tables 2 and 5). This finding could be explained by the fact that urea-derived inhibitors, e.g. 50 and 51, can potentially form an additional hydrogen bond to the target cathepsins, in contrast to the corresponding compounds with a carbamate P3-P2 linker, e.g. 45.

Table 5. $K_i$ values of urea-based compounds 50, 51 and 62–66 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>R$^1$</th>
<th>n</th>
<th>R$^2$</th>
<th>X</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
<td>cath S</td>
</tr>
<tr>
<td>50</td>
<td>H</td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>51</td>
<td>H</td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>0.045 ± 0.002</td>
</tr>
<tr>
<td>62</td>
<td></td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>63</td>
<td></td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>64</td>
<td></td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>65</td>
<td></td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>66</td>
<td></td>
<td>0</td>
<td>H</td>
<td>CH$_2$</td>
<td>4700 ± 400</td>
</tr>
</tbody>
</table>

The inhibitors 62–65 containing extended P3 moieties exhibited similar binding affinities on cathepsin K with picomolar $K_i$ values. Triaryl derivatives 63 and 65 were slightly more potent than the methyl substituted biaryl compounds 62 and 64. Among the six urea-based azadipeptide nitriles, 65 displayed the most promising selectivity profile for cathepsin K over
cathepsins L, S, and B (Table 5). The corresponding carba-analogue 66 showed the same trend to selectively inhibit cathepsin K, but with approximately three orders of magnitude higher $K_i$ value. A fast-binding inhibition behavior was observed for the carba-analogue 66, in contrast to the azadipeptide nitriles 50, 51 and 62–65.

A further shortening of P3-P2 linker of 65 and 66 led to the amide-based dipeptide nitrile 79 and azadipeptide nitrile 67. While the carba-analogue 79 with a fast-binding behavior exhibited a 300–7600-fold selectivity for cathepsin K over cathepsins L and B and a moderate selectivity over cathepsin S, the corresponding azadipeptide nitrile 67, a slow-binding inhibitor, was more potent, but much less selective (Table 6). Interestingly, the methylated counterparts of 79 and 67, compounds 80 and 68, showed a remarkable selectivity for cathepsin K over cathepsins L, S and B. Moreover, compound 68 represents the first cathepsin K-selective azadipeptide nitrile at all. Azadipeptide nitrile 68 still exhibited a picomolar inhibition constant for cathepsin K in combination with a 200–4300-fold selectivity over antitargets, cathepsins L, B, and S. Probably, the hydrogen-bond donating CONH linker of 67 contributes to the binding to the four cathepsins studied whereas the P2 and P3 moieties are already optimized for cathepsin K. Therefore, the disruption of hydrogen bond formation by the methylation a CONH linker is better tolerated by cathepsin K than by the other cathepsins. This suggestion is confirmed by the experimental data.

Table 6. $K_i$ values of amide-based compounds 79, 80, 67 and 68 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$X$</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>79</td>
<td>H</td>
<td>H</td>
<td>CH$_2$</td>
<td>940 ± 40</td>
</tr>
<tr>
<td>80</td>
<td>Me</td>
<td>H</td>
<td>CH$_2$</td>
<td>&gt; 22000</td>
</tr>
<tr>
<td>67</td>
<td>H</td>
<td>Me</td>
<td>NMe</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>68</td>
<td>Me</td>
<td>Me</td>
<td>NMe</td>
<td>2700 ± 300$^a$</td>
</tr>
</tbody>
</table>

$^a$The progress curves were analyzed by linear regression in a time interval between 20 and 30 min.

$^b$The progress curves were followed over 20 min and analyzed by non-linear regression.
While compounds 50, 51 and 62–65 clearly showed slow-binding inhibition type, a fast-binding inhibition behavior was observed for the carba-analogue 66. The \( k_{on} \) values of the latter compounds for the four cathepsins reflect the different \( K_i \) values (for \( k_{on} \) values of 50, 51 and 62–65, see Table 7). Except compound 51, the highest second-order rate constants (\( k_{on} \)) were calculated for association processes between urea-based azadipeptide nitriles and cathepsins S and K. These high \( k_{on} \) values correlate with the corresponding low \( K_i \) values which were obtained for compounds 50 and 62–65 and outline the difficulty to achieve the inhibitor selectivity for cathepsin K over cathepsin S. As a general trend, it was further observed that the introduction of biaryl or triaryl P3 substituents results in decreased \( k_{on} \) values (50 and 51 versus 62–65). This effect was particularly strong in the case of triaryl derivatives 63 and 65 and cathepsin L. Moreover, the phenylurea-derived inhibitors 51, 64 and 65 showed slightly higher second-order association rate constants than the corresponding benzylureas 50, 62 and 63.

Table 7. \( k_{on} \) values of urea-based compounds 50, 51 and 62–65.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>( R^1 )</th>
<th>n</th>
<th>( R^2 )</th>
<th>X</th>
<th>( k_{on} ) (( 10^3 ) M(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>50</td>
<td>H</td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>710 ± 40</td>
</tr>
<tr>
<td>51</td>
<td>H</td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>3400 ± 100</td>
</tr>
<tr>
<td>62</td>
<td>N(\equiv)O N(\equiv)</td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>310 ± 20</td>
</tr>
<tr>
<td>63</td>
<td>N(\equiv)S N(\equiv)</td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>64</td>
<td>N(\equiv)N</td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>250 ± 10</td>
</tr>
<tr>
<td>65</td>
<td>N(\equiv)N</td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>66</td>
<td>H</td>
<td>0</td>
<td>H</td>
<td>CH(_2)</td>
<td>n.d.(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Not determined
In the case of the amide-based azadipeptide nitrile 67, as expected, the highest $k_{on}$ value was calculated for cathepsin K. However, the differences in the second-order rate constants of 67 on cathepsins L, S and B were less pronounced than, e.g., in the case of the urea-derived azadipeptide nitrile 65 containing the same triaryl moiety at the P3 position. Furthermore, the methylation of the P3-P2 linker strongly affected the enzyme-inhibitor association rate (67 versus 68, Table 8). The N-methyl compound 68 showed clearly decreased $k_{on}$ values, reflecting a delayed approach to steady-state. This effect was less pronounced at cathepsin K, for which the optimized substructures, common in 67 und 68, attenuate the lack of a hydrogen bond formation to the backbone amide of Gly66 [164] in the case of 68. The methylation of the P2-P3 amide linker in compound 67, which was applied for the development of cathepsin K inhibitor 68, represents an important new approach to achieve the selectivity of azadipeptide nitriles toward particular cysteine proteases. This approach was first used in the presented work and requires a two step modification of the azadipeptide nitrile scaffold. In the first step, the substitution pattern of the inhibitor molecule had to be optimized for a target cysteine protease. In the second step, the hydrogen bond to the P3-P2 linker was to be disrupted, e.g., by N-alkylation to achieve the selectivity.

Table 8. $k_{on}$ values of amide-based compounds 67 and 68.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>X</th>
<th>$k_{on}$ ($10^3$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>79</td>
<td>H</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.$^a$</td>
</tr>
<tr>
<td>80</td>
<td>Me</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.</td>
</tr>
<tr>
<td>67</td>
<td>H</td>
<td>Me</td>
<td>NMe</td>
<td>800 ± 80</td>
</tr>
<tr>
<td>68</td>
<td>Me</td>
<td>Me</td>
<td>NMe</td>
<td>n.d.$^{a,b}$</td>
</tr>
</tbody>
</table>

$^a$Not determined

$^b$For [68] = 20 µM, a $k_{obs}$ value could not be obtained by non-linear regression of the progress curves. A limit $k_{obs}(1+[S]/K_m)/[I] < 1.0 \times 10^3$ M$^{-1}$s$^{-1}$ was therefore estimated.

$^c$For [68] = 350 nM, a $k_{obs}$ value could not be obtained by non-linear regression. A limit $k_{obs}(1+[S]/K_m)/[I] < 20 \times 10^3$ M$^{-1}$s$^{-1}$ was therefore estimated.

$^d$The progress curves were followed over 20 min and analyzed by non-linear regression.
In contrast to $k_{on}$ values, the corresponding $k_{off}$ values of the urea- and amide-based azadipeptide nitriles were independent from the substitution pattern and the nature of the P3-P2 linker. As shown in Table 9, the first-order rate constants of compounds 50, 51 and 62–65 were approximately in the same range for each enzyme. Furthermore, it could not be observed that the $k_{off}$ values of azadipeptide nitriles 50, 51 and 62–65 significantly differed between cathepsins L, S, K and B. However, the first-order dissociation rate constants of urea-based azadipeptide nitriles and cathepsin K were slightly lower than the corresponding $k_{off}$ values on cathepsins L and S.

Table 9. $k_{off}$ values of urea-based compounds 50, 51 and 62–65.

| cmpd | $R^1$ | $n$ | $R^2$ | $X$ | $k_{off}$ \(10^{-3}\) s$^{-1}$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>50</td>
<td>H</td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>51</td>
<td>H</td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>62</td>
<td></td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>63</td>
<td></td>
<td>1</td>
<td>Me</td>
<td>NMe</td>
<td>0.090 ± 0.009</td>
</tr>
<tr>
<td>64</td>
<td></td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>65</td>
<td></td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>66</td>
<td></td>
<td>0</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.$^a$</td>
</tr>
</tbody>
</table>

$^a$Not determined

While the first-order rate constants of the amide-derived compound 67 were obtained for the four tested cathepsins and showed no significant differences to those of amide-based azadipeptide nitriles, the $k_{off}$ values of $N$-methyl derivative 68 could be calculated only for cathepsins K and B (Table 10). Because the association of compound 68 with cathepsins
L and S was very slow, it was not possible to determine the second-order rate constants from the hyperbolic progress curves for these two enzymes. For this reason, the first-order rate constants of 68 for cathepsins L and S could not be calculated because of $k_{\text{off}} = K_i \times k_{\text{on}}$.

Table 10. $k_{\text{off}}$ values of amide-based compounds 67 and 68.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>R\textsuperscript{1}</th>
<th>R\textsuperscript{2}</th>
<th>X</th>
<th>$k_{\text{off}}$ (10\textsuperscript{-3} s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>79</td>
<td>H</td>
<td>H</td>
<td>CH\textsubscript{2}</td>
<td>n.d.\textsuperscript{a}</td>
</tr>
<tr>
<td>80</td>
<td>Me</td>
<td>H</td>
<td>CH\textsubscript{2}</td>
<td>n.d.</td>
</tr>
<tr>
<td>67</td>
<td>H</td>
<td>Me</td>
<td>NMe</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>68</td>
<td>Me</td>
<td>Me</td>
<td>NMe</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Not determined

\textsuperscript{b}The progress curves were analyzed by non-linear regression in a time interval of 20 min.
2. RESULTS AND DISCUSSION

2.1.7. CONCLUSIONS I

In this study, using the example of cathepsin K, an approach to design of highly potent and selective azadipeptide nitrile inhibitors was demonstrated. Whereas the carba-analogous dipeptide nitriles typically show fast-binding kinetics, a different, slow-binding behavior was observed for azadipeptide nitriles. Thus, it was possible to determine the influence of structural features on association and dissociation rate constants for the presented series of peptidomimetic nitrile inhibitors. A strong impact of structural variations in azadipeptide nitriles on the enzyme-inhibitor association rate was demonstrated. Although reversibly [192] forming isothiosemicarbazide adducts, these compounds gain their inhibitory activity not only from the covalent attraction, but also from specific non-covalent interactions to the active site. The dissociation rate, however, was not affected by the compounds’ structure. This finding reflects the difficulty in delivering significant binding energy from non-covalent interactions, as concluded from the large and relatively shallow active site of cysteine cathepsins.

Furthermore, synthetic routes to urea- and amide-based azadipeptide nitriles were established in the presented study. Compound 68 was identified as the first cathepsin K-selective azadipeptide nitrile with a picomolar $K_i$ value. The selectivity for cathepsin K was achieved by the stepwise optimization of the inhibitor scaffold combined with the methylation of the P3-P2 amide linker. To explain the remarkable selectivity profile of 68 (200–4300-fold) toward cathepsin K, it was suggested that the P3 and P2 substituents in compound 68, which were optimized for cathepsin K, can compensate the negative effect of the hydrogen bound disruption by $N$-methylatation. Therefore, the CONMe linker was better tolerated by cathepsin K than by the other cathepsins. The described approach represents a new method to develop selective azadipeptide nitriles and can eventually be applied for the other cysteine cathepsins.

Moreover, the first example for direct cyclization of a Cbz-protected azadipeptide tert-butyl ester to the corresponding 1,2,4-triazinane-3,6-dione was described. This cyclization reaction represents an easy synthetic access to 1,2,4-triazinane-3,6-dione scaffolds for the preparation of new potential peptidomimetic structure with at least four possible diversity points.

Parts of this study are described in a recently published manuscript by Frizler et al. [192].
2.1.8. HOMOCYCLOLEUCINE-BASED AZADIPEPTIDE NITRILES

As described in chapter 2.1.1, the homocycloleucine-derived azadipeptide nitrile 42 with a P3-P2 carbamate linker was identified as potent cathepsin K inhibitor \( (K_i = 1.8 \text{ nM}) \). Furthermore, this compound was already 72–220-fold selective for cathepsin K over cathepsins L, S and B (Table 2). To improve the potency and selectivity of the azadipeptide nitrile 42 toward cathepsin K, it was decided to combine its P2 substituent with the already for cathepsin K optimized triaryl P3 substituent and the P3-P2 linkers of 65 and 67 (Fig. 30).

In the first attempt to prepare compound 91 (Fig. 30), the following synthetic route was applied. Commercially available homocycloleucine 93 was first converted into the corresponding benzyl ester 94 by the reaction with benzyl alcohol in toluene under Dean-Stark conditions and in the presence of \( p \)-toluenesulfonic acid (Scheme 12).
Next, compound 94 was reacted with 4-cyanophenyl isocyanate in dry THF to obtain the urea derivative 95 in high yield and purity. To transform the nitrile compound 95 to the corresponding N-(thiophene-2-carbonyloxy)benzimidamide 96, it was heated with hydroxylamine hydrochloride in the presence of DIPEA, followed by the reaction with 2-thiophenecarbonyl chloride in the presence of DIPEA. Surprisingly, the desired compound 96 was not formed. The isolated product was characterized as the hydantoin derivative 97 (Scheme 13).

\[ \text{Scheme 13. Unexpected formation of the hydantoin derivative 97 under basic conditions.} \]
a) 4-cyanophenyl isocyanate, TEA, THF, rt; b) NH$_2$OH $\times$ HCl, DIPEA, EtOH, $\Delta$; c) 2-thiophenecarbonyl chloride, DIPEA, MeCN, rt.

It was supposed that the polarization of the N-H-bond of the aryl-substituted urea nitrogen by a strong organic base (DIPEA) caused the nucleophilic attack on the carbonyl carbon of the benzyl ester moiety. The hydantoin ring was formed by the following elimination of the benzyl alcohol. The supposed mechanism was confirmed by the reaction of compound 98 with 1,2-dimethylhydrazine dihydrochloride in the presence of DIPEA (Scheme 14), where the formation of the desired 1,2-dimethylhydrazide 99 could not be annotated. Instead, the hydantoin derivative 100 was isolated as a main product. Interestingly, the formation of a hydantoin was not observed for the transformation of 53 into the corresponding L-leucine-derived N-(thiophene-2-carbonyloxy)benzimidamide 57 (Scheme 5). The high cyclisation tendency of homocycloleucine-derived compounds could be explained by the gem-dialkyl effect which was first described in 1915 by Beesley, Thorpe and Ingold as part of a study on the formation and stability of spiro compounds [193]. These authors showed that the
increasing size of two substituents on a tetrahedral center leads to enhanced reactivity between parts of the other two residues for intramolecular reactions. The first explanation for the \textit{gem}-dialkyl effect was the internal angle reduction and is commonly referred to as the ‘Thorpe-Ingold effect’ or the ‘theory of valency deviation’. Because the theory of valency deviation is not generally valid, it was later extended by further theories. For example, the ‘reactive-rotamer hypothesis’ expects the \textit{gem}-disubstitution to promote the formation of reactive gauche conformers, in which the functionalities are located syn-periplanar to each other, as a possible reason for the enhanced cyclisation tendency (for reviews, see [194], [195]).

![Reaction scheme](image)

**Scheme 14.** Reaction of 98 with (NHMe)$_2$ $\times$ 2HCl in the presence of DIPEA.

a) 4-cyanophenyl isocyanate, DIPEA, THF, rt; b) (NHMe)$_2$ $\times$ 2HCl, DIPEA, THF, rt;

In contrast to the L-leucine-based compounds, the linear synthesis of the homocycloleucine-derived azadipeptide nitrile 91 (Fig. 30) was not successful due to the cyclisation reaction described in Scheme 13. Therefore, a convergent synthetic route was developed. As shown in Figure 31, compound 91 was retrosynthetically divided into the synthetic equivalents 101, 94 and 102. The synthetic equivalents 101 and 102 had to be synthesized, while the P2 building block 94 was already prepared (Scheme 12). In the first step of the envisaged synthetic route, the P3 building block 101 was to be coupled with the P2 building block 94 using the Lossen rearrangement leading to the corresponding urea-derived intermediate. In the second step, the benzyl ester of the obtained intermediate was to be cleaved \textit{via} a catalytic hydrogenation on Pd/C, followed by the coupling of the resulting acid with 102 using the mixed anhydride.
procedure. The mixed anhydride protocol allows gentle coupling in the absence of strong bases, which is important to avoid the possible intramolecular cyclisation.

![Chemical structure](image1)

**Figure 31.** Retrosynthetic route to compound 91.

The hydroxamic acid 101 was synthesized as the P3 building block as shown in Scheme 15. The carboxylic group of the triaryl derivative 69 (Scheme 7) was activated with isobutyl chloroformate to a mixed anhydride and reacted with hydroxylamine [196] to obtain compound 101. The crude product could be easily purified by a simple recrystallization from ethanol/ethyl acetate. Next, the hydroxamic acid 101 was converted into the corresponding isocyanate by the reaction with EDC in the presence of DMAP via Lossen rearrangement [197] and reacted *in situ* with the P2 building block 94 to obtain the urea-based intermediate 103 in high yield and purity.

![Chemical structure](image2)

**Scheme 15.** Synthesis of compound 101 as the P3 building block and its coupling to intermediate 103.

- a) 1. ClCO$_2$-Bu, NMM, THF, -25 °C, 2. NH$_2$OH × HCl, NaOH, MeOH, THF, -25 °C to rt;
- b) 1. EDC, DMAP, THF, rt; 2. 94 after basic extraction, THF, rt.
In the next step, it was tried to cleave the benzyl ester of 103 via catalytic hydrogenation on Pd/C to obtain the free acid 104 for the further coupling with 1,2-dimethylhydrazinecarbonitrile 102. Unfortunately, the hydrogenolytic cleavage of the benzyl ester of 103 was not successful. The isolated product, which was purified by column chromatography, showed a mass-to-charge ratio value (m/z) of 505.3 ([M + H]+) in the recorded MS(ESI) spectrum. In combination with NMR spectra, this finding indicated the catalytic hydrogenation of the 1,2,4-oxadiazole ring to the corresponding dihydro-1,2,4-oxadiazole derivative 105 instead of deprotection of the carboxylic group (Scheme 16). However, the location of the double bond within the formed dihydro-1,2,4-oxadiazole heterocycle was not clearly resolved and has to be clarified by further experiments.


a) H₂, Pd/C, MeOH, rt, atmospheric pressure; X = dihydro-1,2,4-oxadiazole ring.

To realize a new synthetic strategy, the desired compound 91 was again retrosynthetically divided into three synthetic equivalents (compounds 101, 106, and BrCN, Fig. 32).

Figure 32. New retrosynthetic route to compound 91.
This synthetic route was first applied in a modified form to the carba-analogues 110 and 111 (Scheme 18). Boc-protected homocycloleucine 107 was activated with isobutyl chloroformate to a mixed anhydride followed by the reaction with aminoacetonitrile monosulfate in the presence of TEA. The obtained dipeptide nitrile 108 was treated with methanesulfonic acid to cleave the Boc protecting group. The basic extraction of the resulting methanesulfonate salt led to compound 109 as the P2 building block (Scheme 17).

\[ \begin{align*} 
\text{Scheme 17. Synthesis of the P2 building block 109.} \\
a) &\quad \text{(Boc)}_2\text{O, NaOH, dioxane, rt;} \\
b) &\quad 1. \text{ClCOC}_2\text{-Bu, NMM, THF, -25 °C, 2. H}_2\text{NCH}_2\text{CN × H}_2\text{SO}_4, \text{TEA, THF, -25 °C to rt;} \\
c) &\quad \text{MeSO}_3\text{H, THF, rt, basic extraction.} 
\end{align*} \]

Next, the obtained P2 building block 109 was reacted via Lossen rearrangement with the P3 building block 101. Unfortunately, this reaction was not successful, and the formation of the desired compound 110 could not be observed. However, the reaction of compound 109 with the P3 building block 69 led to the formation of the corresponding dipeptide nitrile 111 containing a P3-P2 amide linker (Scheme 18).

\[ \begin{align*} 
\text{Scheme 18. Planned synthetic route to compound 110 (above) and synthesis of dipeptide nitrile 111.} \\
a) &\quad \text{EDC, DMAP, 109, THF, rt.} 
\end{align*} \]

Due to the comparable kinetic profiles of urea- and amide-derived azadipeptide nitriles (65 versus 67), it was decided not to further attempt preparing urea-based cathepsin inhibitors containing homocycloleucine at the P2 position. For reasons of synthetic access, the P3-P2
2. RESULTS AND DISCUSSION

Amide linker was maintained in all compounds of this study. In Figure 33, the final retrosynthetic route to the amide-based azadipeptide nitrile 92 is shown. While cyanogen bromide was commercially available and compound 69 was already obtained (Scheme 7), the P2 building block 106, containing a gem-disubstituted amino acid (homocycloleucine), had to be synthesized.

![Figure 33. Retrosynthetic route to the amide-based azadipeptide nitrile 92.](image)

To prepare the P2 building block 106, it was first tried to improve the coupling of Cbz-protected homocycloleucine 39 (Scheme 2) with 1,2-dimethylhydrazine. When compound 39 was reacted with oxalyl chloride for activation, the N-carboxyanhydride (NCA) 113 was obtained in a yield of 83% (Scheme 19). Its formation was envisaged to occur via nucleophilic attack of the carbamate oxygen at the activated carboxylic group to produce the 5(4H)-oxazolone 112. Under the used conditions in the absence of a base [198], a chloride-promoted displacement of the benzyl group was operative [199].

![Scheme 19. Synthesis of the homocycloleucine-NCA 113.](image)

a) (COCl)$_2$, CH$_2$Cl$_2$, DMF, rt.

The obtained Leuchs anhydride 113 was reacted with 1,2-dimethylhydrazinecarbonitrile 102 (Fig. 31) in order to obtain the azadipeptide nitrile 114 as a building block (Scheme 20) which could have been directly coupled with compound 69 leading to the desired product 92 in only one further step. However, the transformation of 113 into 114 was not observed under
different conditions, ranging from a reaction in THF at room temperature to a reaction in DMF at 130 °C in a sealed tube. In contrast, the transformation of 113 with aminoacetonitrile in THF carried out in an autoclave at 100°C, provided homocycloleucyl-glycine-nitrile 109 in 72%. Thus, two alternative routes from homocycloleucine 93 to the P2 building block 109 (via 107 and 108, or via 39 and 113) were employed.

![Scheme 20. Reaction of 113 with different nucleophiles. Box. Synthesis of 102.](image)
a) BrCN, H₂O, NaOH, rt; b) 102, THF, rt; c) 102, DMF, 130 °C; d) N₂NCH₂CN × H₂SO₄, THF, DIPEA, 100 °C.

The unexpectedly [200] low reactivity of the homocycloleucine-derived NCA 113 was considered to result from the gem-dialkyl effect. It has been well established that this effect promotes cyclisation reactions due to an increased number of gauche interactions in the open chain substrates and/or a relief of steric strain in the cyclic products (for reviews, see [194], [195]). However, the gem-dialkyl effect also leads to an enhanced stability of the corresponding cyclic compounds, and it was therefore assumed that it accounts for the protection of 113 against hydrazinolytic cleavage. As noted above, the mixed anhydride coupling of Cbz-protected homocycloleucine 39 took place with low yields for reasons of steric interference (Scheme 2). Additionally, these reactions might be impaired by the gem-dialkyl effect leading to the formation of heterocyclized compound(s).

In further attempts to improve the yield of the dimethylhydrazide 40, the EDC/DMAP and HBTU/HOBt coupling protocols were used (see also the ‘Experimental Section’). In the first experiment, the Cbz-protected homocycloleucine 39 was activated with EDC in the presence of DMAP, treated with triethylamine and 1,2-dimethylhydrazine dihydrochloride and stirred at room temperature for 24 h to obtain 40 in yield of 36% (18% in the case of the mixed
anhydride method, see also Scheme 2). When the reaction mixture was alternative heated to reflux for 24 h, the yield of 40 was slightly improved to 39%. The HBTU/HOBt protocol offered no further synthetic advantages. Compound 40 was isolated only in a yield of 29% when 39 was activated with HBTU in the presence of HOBt, treated with triethylamine and 1,2-dimethylhydrazine dihydrochloride, and stirred for 3 d at room temperature. In general, it was not possible to significantly improve the yield of 1,2-dimethylhydrazide derivative 40 due to the already discussed gem-dialkyl effect. The EDC/DMAP protocol in combination with the heating of the reaction mixture represents the best coupling procedure to prepare the 1,2-dimethylhydrazide 40 (Scheme 21).

![Scheme 21](image)

**Scheme 21.** Synthesis of 40 using different coupling procedures.

a) 1. EDC, DMAP, THF, rt; 2. (NHMe)$_2$×2HCl, TEA, rt, 24 h; 36%; b) 1. EDC, DMAP, THF, rt; 2. (NHMe)$_2$×2HCl, TEA, Δ, 24 h; 39%, c) 1. HBTU, HOBt, THF, rt; 2. (NHMe)$_2$×2HCl, TEA, rt, 3 d; 29%.

Before starting the final synthetic sequence to the desired amide-based azadipeptide nitrile 92, compound 40 was reacted with (Boc)$_2$O in the presence of DMAP for the confirmation of the supposed mechanism of the 1,2,4-triazinane-3,6-dione formation described in chapter 2.1.4, Scheme 9. The resulting product 115 was characterized as an unique spiro compound with 1,3,4-triazaspiro[5.5]undecan-2,5-dione scaffold (Scheme 22).

![Scheme 22](image)

**Scheme 22.** Formation of 115 by the reaction with (Boc)$_2$O in the presence of DMAP.

a) (Boc)$_2$O, DMAP, MeCN, rt.

The finally successful synthesis of compound 92, the aza-analogue of 111, is outlined in Scheme 23. The Cbz protecting group of the dimethylhydrazide 40 was hydrogenolytically removed to obtain the building block 106. The synthetic equivalent 69 (Fig. 33) was activated with EDC in the presence of DMAP and reacted with 106 leading to the formation of
compound 116. In the final reaction step with cyanogen bromide, the homocycloleucine-based azadipeptide nitrile 92, bearing a large P3 substituent, was obtained.

![Chemical structure](image)

**Scheme 23.** Synthesis of the amide-based azadipeptide nitrile 92.

a) Pd/C, H₂, MeOH, rt; b) 69, EDC, DMAP, CH₂Cl₂, rt; c) BrCN, NaOAc, MeOH, rt.
2.1.9. KINETIC CHARACTERISATION OF 43, 111 AND 92

The product 92, as well as its carba-analogue 111, were tested on the human cathepsins L, S, K and B (Table 11). Again, the azadipeptide nitrile 92 showed a slow-binding behavior and a lower $K_i$ value than the carba-analogue 111, a fast-binding inhibitor. A comparison of the two aza derivatives 92 and 42 revealed that the introduction of a large substituent in the P3 position as well as the replacement of the P3-P2-linker was advantageous and led to an improved potency and selectivity for cathepsin K. The same trend was also observed for the corresponding dipeptide nitriles 43 and 111. The improved inhibitory activity of the triaryl derivatives 111 and 92, compared with their monoaryl counterparts 42 and 43, could be explained by enhanced hydrophobic interactions between 111 and 92 and the tested cathepsins (particularly cathepsin K) due to the increased molecular weight and lipophilicity. Interestingly, the homocycloleucine moiety at the P3 position alone seems to be a sufficient structural feature to develop cathepsin K-selective azadipeptide nitriles, while the L-leucine-derived azadipeptide nitriles requires the methylation of the P3-P2 amide linker to achieve the selectivity for cathepsin K over cathepsins L, S and B (68 versus 92). Furthermore, compound 92 was slightly more potent on cathepsin K ($K_i = 0.35 \text{ nM}$) than the corresponding $N$-methylated counterpart 68 ($K_i = 0.63 \text{ nM}$) and less potent but much more selective than the non-methylated L-leucine-derived azadipeptide nitrile 67 ($K_i = 0.032 \text{ nM}$).

Table 11. $K_i$ values of compounds 42, 43, 111 and 92 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$X$</th>
<th>$R^2$</th>
<th>$Y$</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>42</td>
<td>H</td>
<td>CH$_2$O</td>
<td>Me</td>
<td>NMe</td>
<td>400 ± 50</td>
</tr>
<tr>
<td>43</td>
<td>H</td>
<td>CH$_2$O</td>
<td>H</td>
<td>CH$_2$</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>111</td>
<td>S</td>
<td>H</td>
<td>CH$_2$</td>
<td></td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>92</td>
<td>S</td>
<td>Me</td>
<td>NMe</td>
<td></td>
<td>280 ± 50$^b$</td>
</tr>
</tbody>
</table>

$^a$The kinetic data of compound 42 are shown here for reasons of better illustration and are also depicted in Table 2. $^b$The enzymatic reaction was started by addition of chromogenic substrate after the enzyme was preincubated with inhibitor for 30 min. The reaction lines were analyzed by linear regression.
Additionally, the analysis of the progress curves in the case of slow-binding inhibitors 42 (see also Table 3) and 92 allowed for the determination of the second-order association rate constants ($k_{\text{on}}$) and the corresponding first-order dissociation rate constants ($k_{\text{off}}$). The results are shown in Tables 12 and 13. Because it was not possible to obtain the first-order rate constant ($k_{\text{obs}}$) for the development of the steady-state equilibrium between compound 92 and cathepsin L by non-linear regression, a limit $k_{\text{obs}}(1+[S]/K_m)/[I] < 10 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ was estimated for $[92] = 5 \mu\text{M}$. In general, the obtained $k_{\text{on}}$ values of compounds 42 and 92 correlate with the corresponding $K_i$ values (Tables 11 and 12). Furthermore, the estimated $k_{\text{on}}$ values were clearly influenced by homocycloleucine/L-leucine replacement. Except of the $N$-methylated derivative 68, the L-leucine-containing inhibitors 45, 50, 51, 62–65 and 67 exhibited greater $k_{\text{on}}$ values than 42 and 92 on the four tested cathepsins (Tables 3, 7, 8 and 12). The second-order association rate constants of 42 and 92 on cathepsin K were less affected than those on cathepsins L, S and B by the introduction of the homocycloleucine moiety into the azadipeptide nitrile scaffold. These results reflect the selectivity of homocycloleucine-derived azadipeptide nitriles for cathepsin K over the antitargets, cathepsins L, S and B.

Table 12. $k_{\text{on}}$ values of compounds 42 and 92 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$X$</th>
<th>$R^2$</th>
<th>$Y$</th>
<th>$k_{\text{on}}$ ($\times 10^3 \text{ M}^{-1} \text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>42</td>
<td>H</td>
<td>CH$_2$O</td>
<td>Me</td>
<td>NMe</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>43</td>
<td>H</td>
<td>CH$_2$O</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.</td>
</tr>
<tr>
<td>111</td>
<td>0</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>92</td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>n.d.$^b,c$</td>
<td>4.8 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$The kinetic data of compound 42 are shown herein for reasons of better illustration and also depicted in Table 3.

$^b$Not determined.

$^c$For $[92] = 5 \mu\text{M}$, a $k_{\text{obs}}$ value could not be obtained by non-linear regression. Therefore, a limit $k_{\text{obs}}(1+[S]/K_m)/[I] < 10 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ was estimated.

As shown in Table 13, the calculated first-order dissociation rate constants ($k_{\text{off}}$) of 42 and 92 were in the same range, and showed to be independent from the structural modification.
of the P3 substituent and the P3-P2 linker. Furthermore, the \( k_{\text{off}} \) values were not affected by homocycloleucine/L-leucine replacement (e.g. 92 versus 67).

Table 13. \( k_{\text{off}} \) values of compounds 42 and 92 on human cathepsins L, S, K and B

| cmpd | R\(^1\) | X | R\(^2\) | Y | \( k_{\text{off}} \) (\( \times 10^{-3} \) s\(^{-1}\))
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>42(^a)</td>
<td>H</td>
<td>CH(_2)O</td>
<td>Me</td>
<td>NMe</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>43</td>
<td>H</td>
<td>CH(_2)O</td>
<td>H</td>
<td>CH(_2)</td>
<td>n.d.(^b)</td>
</tr>
<tr>
<td>111</td>
<td></td>
<td>0</td>
<td>H</td>
<td>CH(_2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>92</td>
<td></td>
<td>0</td>
<td>Me</td>
<td>NMe</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(^a\)The kinetic data of compound 42 are shown here for reasons of better illustration and are also depicted in Table 4.
\(^b\)Not determined.
2. RESULTS AND DISCUSSION

2.1.10. FLUORESCENT AZADIPEPTIDE NITRILES

To study the ability of azadipeptide nitriles to penetrate the cell membrane as well as to investigate their cell distribution and accumulation, it is important to have pharmacological tools which allow for visualization of these compounds. Therefore, it was decided to introduce a fluorescent reporter in the homocycloleucyl-methylazaalanine nitrile scaffold of the cathepsin K-selective inhibitor 92 to obtain a VIS-detectable homocycloleucine-based azadipeptide nitrile.

At first, the fluorescent dipeptide nitriles 119 and 120 were synthesized, as shown in Scheme 24, to study their kinetic profiles on cathepsins L, S, K and B. For the labeling of the P3 position, two different fluorophores, compounds 117 and 118 (Fig. 34), were used. While dansyl chloride 117 was commercially available, the coumarin derivative 118 was synthesized by Matthias Mertens as previously described [202].

![Figure 34. Compounds 117 and 118 as P3 fluorophores.](image)

The Cbz-protected homocycloleucine 39 was reacted with oxalyl chloride in the presence of a catalytic amount of DMF to obtain homocycloleucine-NCA 113 in high yield and purity. Compound 113 was further converted with aminoacetonitrile leading to the dipeptide nitrile 109. Finally, the free amino group of 109 was reacted with dansyl chloride 117 and the coumarin derivative 118 to obtain the fluorescent labeled dipeptide nitriles 119 and 120, respectively. As expected, the reactivity of the amino group of 109 was decreased due to the gem-disubstitution of the α-carbon of the homocycloleucine at the P2 position (see also chapter 2.1.8). Therefore, the target compounds 119 and 120 were isolated by chromatographic separation only in low yields of 36% and 20%, respectively.

Due to the better kinetic properties of the dipeptide nitrile 120 compared with the corresponding dansyl derivative 119, it was decided to introduce the coumarin fluorophore 118 into the azadipeptide nitrile scaffold of compound 92. The synthetic route to the first fluorescent homocycloleucine-based azadipeptide nitrile 122 is shown in Scheme 25.
Scheme 24. Synthesis of the fluorescent labeled dipeptide nitriles 119 and 120.

a) (COCl)$_2$, CH$_2$Cl$_2$, DMF, rt; b) N$_2$NCH$_2$CN × H$_2$SO$_4$, THF, DIPEA, 100 °C; c) 117, TEA, THF, rt then Δ; d) 118, EDC, DIPEA, THF, rt.

Cbz-protected compound 40 was hydrogenated on Pd/C to obtain 106. The carboxylic group of the P3 fluorophor 118 was activated with EDC and reacted with the P2 building block 106 leading to the 1,2-dimethylhydrazide derivative 121. Finally, compound 121 was converted into the corresponding azadipeptide nitrile 122 by the reaction with cyanogen bromide.

Scheme 25. Synthesis of the fluorescent labeled azadipeptide nitrile 122.

a) Pd/C, H$_2$, MeOH, rt; b) 118, EDC, DMAP, THF, rt; c) BrCN, NaOAc, MeOH, rt.
2.1.11. KINETIC CHARACTERISATION OF 119, 120 AND 122

The obtained fluorescent compounds 119, 120 and 122 were tested on cathepsins L, S, K and B. While the dipeptide nitriles 119 and 120 showed a fast-binding behavior, theaza-analogous counterpart 122 was, as expected, a slow-binding inhibitor. The fluorescent probe 120 with the N-(7-diethylamino)-2-oxo-2H-chromene-3-carbonyl moiety at the P3 position was a better cathepsin K inhibitor than the corresponding dansyl derivative 119 (Table 14). Furthermore, the replacement of the α-carbon in compound 120 by a nitrogen atom, leading to the azadipeptide nitrile 122, resulted in an improvement of the inhibitory activity. However, compared with compound 92, the introduction of the P3 building block 118 into the homocycloleucyl-methyazaalanine nitrile scaffold led to an approximately 50-fold weaker cathepsin K inhibitor 122 (0.35 nM versus 19 nM). The fluorescent probe 122 showed a moderate selectivity for cathepsin K over cathepsins L and S (approximately 40-fold), and was only 7-fold selective for cathepsin K over cathepsin B.

Table 14. $K_i$ values of compounds 119, 120, and 122 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$Y$</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>119</td>
<td></td>
<td>H</td>
<td>CH$_2$</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>120</td>
<td></td>
<td>H</td>
<td>CH$_2$</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>122</td>
<td>Me</td>
<td>NMe</td>
<td></td>
<td>0.76 ± 0.08$^a$</td>
</tr>
</tbody>
</table>

$^a$The enzymatic reaction was started by addition of chromogenic substrate after the enzyme was preincubated with inhibitor for 30 min. The reaction lines were plotted by linear regression.

The $k_{on}$ values of compound 122 were different reflecting the corresponding $K_i$ values (Table 15). The highest second-order association rate constant of 122 was calculated for cathepsin K.
2. RESULTS AND DISCUSSION

Table 15. $k_{on}$ values of compound 122 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$Y$</th>
<th>$k_{on}$ ($\times 10^3$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>120</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>122</td>
<td>Me</td>
<td>NMe</td>
<td>n.d.</td>
<td>0.49 ± 0.09</td>
</tr>
</tbody>
</table>

*a* Not determined.

*b* For [122] = 30 µM, a $k_{on}$ value could not be obtained by non-linear regression. Therefore, a limit $k_{on}(1+[S]/K_{m})/[I] < 1.0 \times 10^3$ M$^{-1}$ s$^{-1}$ was estimated.

In contrast to $k_{on}$ values, the first-order rate constants ($k_{off}$) of 122 were, as expected, approximately in the same range (Table 16).

Table 16. $k_{off}$ values of compound 122 on human cathepsins L, S, K and B.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$Y$</th>
<th>$k_{off}$ ($\times 10^3$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>120</td>
<td>H</td>
<td>CH$_2$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>122</td>
<td>Me</td>
<td>NMe</td>
<td>n.d.</td>
<td>0.37 ± 0.07</td>
</tr>
</tbody>
</table>

*a* Not determined.
2. RESULTS AND DISCUSSION

2.1.12. SPECTRAL PROPERTIES OF 122

The absorption and emission spectra of the fluorescence-labeled azadipeptide nitrile 122 were recorded in different solvents to study its spectral properties. The absorption maxima of 122 in dichloromethane, methanol, and water were 422, 424, and 434 nm, respectively. The corresponding emission spectra were 460 nm (dichloromethane), 472 nm (methanol), and 486 nm (water). In general, a slight bathochromic shift of absorption and emission maxima was observed in polar media such as methanol and water. The Stokes shift of 122 in dichloromethane was 38 nm (Fig. 35a). Furthermore, the fluorescence of compound 122 was clearly quenched by methanol and water (Fig. 35b).

![Figure 35](image.png)

**Figure 35.** Spectral properties of the azadipeptide nitrile 122 in different solvents. (a) Excitation (*) and emission (*) spectrum of 122 in CH₂Cl₂; (b) Emission spectra of 122 in CH₂Cl₂ (*), MeOH (*), and H₂O (*).

**Table 17.** Absorption and emission maxima of compound 122.

<table>
<thead>
<tr>
<th></th>
<th>CH₂Cl₂</th>
<th>EtOH</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ_ex (nm)</td>
<td>422</td>
<td>424</td>
<td>434</td>
</tr>
<tr>
<td>λ_em (nm)</td>
<td>460</td>
<td>472</td>
<td>486</td>
</tr>
</tbody>
</table>
2.1.13. CONCLUSIONS II

In this study, a synthetic route to homocycloleucine-derived azadipeptide nitriles was described for the first time. In contrast to azadipeptide nitriles containing a P2 leucine residue, the synthesis of the corresponding homocycloleucine-based inhibitors was considerably more complicated. Herein, the gem-dialkyl effect was discussed as a possible reason for the decreased reactivity of homocycloleucine derivatives for the intramolecular reaction as well as their improved tendency for cyclisation reactions. This effect was particularly strong when 1,2-dimethylhydrazine, a rather weak, but steric demanding nucleophile was used to obtain the desired aza-analogous nitrile inhibitors.

Furthermore, homocycloleucine was confirmed to be a particularly suitable building block for the design of cathepsin K inhibitors. The incorporation of this amino acid into the azadipeptide nitrile scaffold afforded remarkable selectivity and, when combined with the triaryl motif at the P3 position, excellent inhibitory potency. In contrast to the amide-based L-leucine-derived azadipeptide nitriles, the methylation of the P3-P2 amide linker was not necessary to achieve cathepsin K selectivity.

The possible determination of association and dissociation rate constants in the azadipeptide nitrile series enabled us to estimate the influence of a homocycloleucine/L-leucine replacement on these parameters. Whereas $k_{off}$ values were not affected, the L-leucine-containing inhibitors exhibited greater $k_{on}$ values than their homocycloleucine-derived counterparts. Moreover, the second-order association rate constants for cathepsin K were less affected by the homocycloleucine/L-leucine replacement than those for cathepsins L, S and B reflecting the cathepsin K-selectivity of homocycloleucine-derived azadipeptide nitriles.

Moreover, a fluorescence-labeled cathepsin K inhibitor with an azadipeptide scaffold was synthesized, and its kinetic and spectral properties were studied. A coumarin fluorophore was introduced in the homocycloleucine-methylazaalanine-nitrile scaffold, and the fluorescent azadipeptide nitrile 122 was obtained.

Parts of this study are described in a recently published manuscript by Frizler et al. [201].
2. RESULTS AND DISCUSSION

2.2. CATHEPSIN S-SELECTIVE NITRILE INHIBITORS

In previous studies, it was described that the combination of a large group at the P2 position with a small P3 substituent is advantageous for the selective inhibition of cathepsin S (for a review, see [178]). Furthermore, a sulfone within the P2 substituent was proved to be a suitable moiety [165] (Fig. 11). However, it remained unclear, whether cathepsin S can accept larger biaryl und fused aromatic substituents at the P3 position. To clarify this question, it was decided to synthesize a small library of dipeptide nitriles, in which the isobutylsulfone moiety was to be maintained at the P2 position, and various aromatic substituents had to be introduced in the P3 position. It was further envisaged to evaluate the synthesized compounds on cysteine cathepsins L, S, K and B. Moreover, a dipeptide nitrile, exhibiting the best activity/selectivity profiles for cathepsin S, had to be transformed into the corresponding azadipeptide nitrile by Co/N replacement (Fig. 36).

![Figure 36. Optimization of the P3 substituent and the transformation into an azadipeptide nitrile.](image)

R_x = various aryl, biaryl and fused aromatic P3 substituents; R_best = a P3 substituent, whose introduction into the dipeptide nitrile scaffold improves the activity against cathepsin S and enhances the selectivity for this enzyme over antitargets, cathepsins L, K and B.

To obtain dipeptide nitriles with different P3 substituents, the P2 building block 128 was synthesized as shown in Scheme 26. The thiol group of the commercially available Boc-protected cysteine methyl ester 123 was alkylated with isobutyl bromide in the presence of sodium methanolate to obtain the thioether derivative 124. The thioether group of 124 was further oxidized with MCPBA leading to the corresponding sulfone 125. The methyl ester was cleaved under basic conditions, and compound 126 was obtained as a free acid. In the next step, the carboxylic group of 126 was coupled with aminoacetonitrile via a mixed anhydride leading to the Boc-protected dipeptide nitrile 127. Finally, the Boc protecting group of 127 was cleaved with methanesulfonic acid to obtain compound 128 as the
P2 building block. The amino group of 128 was further coupled with various P3 substituents to obtain target dipeptide nitriles 129–138 (Scheme 27). The compounds 129–138 were synthesized by Janina Schmitz in the course of her master thesis under my supervision.

Scheme 26. Synthesis of compound 128 as the P2 building block.
(a) isobutyl bromide, NaOMe, MeOH, ∆; (b) MCPBA, CH₂Cl₂, rt; (c) LiOH, THF/H₂O, rt; (d) 1. TEA, ClCO₂-i-Bu, THF, -25 °C; 2. H₂NCH₂CN × H₂SO₄, THF, rt; (e) MeSO₃H, THF, rt.

Compounds 129 and 130 were obtained by the reaction of the P2 building block 128 in the presence of TEA with benzoyl chloride and thiophene-2-carbonyl chloride, respectively. In contrast, dipeptide nitriles 131–138 were synthesized by the coupling of 128 with the corresponding P3 building blocks using EDC/DMAP protocol (Scheme 27). While the P3 building blocks in compounds 129–134 were commercially available, those of 135–138 were synthesized by Janina Schmitz under my supervision.

a) RCOCl, TEA, THF, rt, 129, R = phenyl, 130, R = 2-thienyl; b) RCOOH, EDC, DMAP, TEA, THF, rt; 131, R = 4-(2-thienyl)phenyl; 132, R = 5-(2-thienyl)-2-thienyl; 133, R = benzo[b]thiophen-2-yl; 134, thieno[2,3-b]thiophen-2-yl; 135, R = 5-(phenyl)-2-thienyl; 136, R = 4-(phenyl)-2-thienyl, 137, R = 5-(3-thienyl)-2-thienyl, 138, R = 5-(1H-tetrazole-5-yl)-2-thienyl.
2. RESULTS AND DISCUSSION

Although Perrey et al. [206] described the alkylation of the N-acyl-cysteine in the presence of sodium methanolate without racemisation, dipeptide nitriles 129–138 were obtained in a racemic form, as it was exemplary shown for compound 129 using chiral HPLC (Fig. 37).

![Chiral Chromatogram of Compound 129](image)

**Figure 37.** The chiral chromatogram of compound 129.

*Column:* LiChroCART® 250-4 ChiraDex® (5 µm); *Conditions:* 95% H₂O/5% MeOH, run time – 20 min, flow – 1 mL/min, UV detector – 214 nm, temperature – 30 °C; *Enantiomeric ratio* = 53:47.

Generally, the presented synthetic route to the P2 building block 128 (Scheme 26) contains two steps with a high risk for racemisation: (1) alkylation of the thiol group of the cysteine derivative 123 in the presence of sodium methanolate, and (2) the cleavage of the methyl ester of 125 with lithium hydroxide.

To prevent the racemisation, a changed synthetic route was applied (Scheme 28) [207]. In this route, the thiol group of L-cysteine 139 was alkylated in the presence of a weaker base, sodium hydroxide, followed by the protection of the amino group using (Boc)₂O to obtain compound 140. To avoid the complicated purification, the thioether derivative 140 was oxidized with KMnO₄ instead of MCPBA (Scheme 26) leading to the corresponding sulfone 141 in high yield and purity. The sulfone derivative 141 was further coupled with aminoacetonitrile via a mixed anhydride to form the Boc-protected dipeptide nitrile 142. Finally, the Boc protecting group of 142 was cleaved with methanesulfonic acid, and the P2 building block 143 was obtained. Because the racemic benzoyl derivative 129 (Scheme 27) showed the best potency and selectivity profiles for cathepsin S, it was decided to introduce the benzoyl substituent into the synthesized P2 building block 143 to obtain the dipeptide nitrile 144 predominantly as R-enantiomer. The enantiopurity of 144 was shown by chiral HPLC on LiChroCART® 250-4 ChiraDex® (5 µm) column using the isocratic elution with
H$_2$O/MeOH (95:5) and a flow of 1 mL/min (Fig. 38). A 1 mM solution of compound 144 in MeCN was injected into the HPLC. The resulting chromatogram showed only one peak (10.7 min) indicating the full enantiomeric purity of 144.

Scheme 28. Synthesis of compound 144 without racemisation.
(a) 1. isobutyl bromide, NaOH, EtOH, rt; 2. (Boc)$_2$O, NaOH, EtOH, rt; (b) KMnO$_4$, AcOH, H$_2$O, rt; (c) 1. TEA, ClCO$_2$-Bu, THF, -25 °C; 2. H$_2$NCH$_2$CN × H$_2$SO$_4$, THF, rt; (d) MeSO$_3$H, THF, rt; (e) benzoyl chloride, DIPEA, THF, rt.

The peak of the enantiopure compound 144 at 10.7 min (Fig. 38) corresponds to the first peak of the racemic dipeptide nitrile 129 (10.8 min, Fig. 37) under used chromatographic conditions and represents the R-enantiomers in both chiral chromatograms.

Figure 38. The chiral chromatogram of compound 144.
Column: LiChroCART® 250-4 ChiraDex® (5 µm); Conditions: 95% H$_2$O/5% MeOH, run time – 20 min, flow – 1 mL/min, UV detector – 214 nm, temperature – 30 °C; Enantiomeric ratio = 100.
In a further experiment (Fig. 39), the 1 mM solutions of 129 and 144 in MeCN were mixed (1:1), and the resulting mixture was injected into the HPLC. Compared with the chiral chromatogram of 129 (Fig. 37), a 25% increase of the AUC was observed for the R-enantiomer peak (10.7 min). The corresponding peak of the S-enantiomer (12.2 min) decreases for the same amount of 25%.

Figure 39. The chiral chromatogram of a 1:1 mixture of compounds 129 and 144.

Column: LiChroCART® 250-4 ChiraDex® (5 µm); Conditions: 95% H₂O/5% MeOH, run time – 20 min, flow – 1 mL/min, UV detector – 214 nm, temperature – 30 °C; Enantiomeric ratio = 78:22.

To synthesize the azadipeptide nitrile 148 (Scheme 29), compound 141 was converted in the 1,2-dimethylhydrazide 145 which was further deprotected to obtain 146. Compound 146 was reacted with benzoyl chloride and cyanogen bromide leading to the azadipeptide nitrile 148.


(a) 1. NMM, ClCO₂-i-Bu, THF, -25 °C; 2. (NHMe)₂ × 2HCl, NaOH, H₂O, to rt; (b) AcCl, EtOH, MeCO₂Et, rt, basic extraction; (c) benzoyl chloride, DIPEA, THF, rt; (d) BrCN, NaOAc, MeOH, rt.
2.2.1. KINETIC CHARACTERISATION OF 129–138 AND 144, 148

The racemic dipeptide nitriles 129–138 and the enantiopure benzoyl derivative 144 as well as the azadipeptide nitrile 148 were tested on human cathepsins L, S, K and B (Table 18 and 19). The kinetic investigations of compounds 129–138 were partially carried out by Janina Schmitz in her master thesis under my supervision.

Table 18. $K_i$ values of target compounds 129–138.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>R</th>
<th>$K_i$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cath L</td>
</tr>
<tr>
<td>129</td>
<td></td>
<td>&gt; 40$^b$</td>
</tr>
<tr>
<td>130</td>
<td></td>
<td>&gt; 40</td>
</tr>
<tr>
<td>131</td>
<td></td>
<td>&gt; 4$^c$</td>
</tr>
<tr>
<td>132</td>
<td></td>
<td>&gt; 4</td>
</tr>
<tr>
<td>133</td>
<td></td>
<td>&gt; 4</td>
</tr>
<tr>
<td>134</td>
<td></td>
<td>&gt; 4</td>
</tr>
<tr>
<td>135</td>
<td></td>
<td>&gt; 4</td>
</tr>
<tr>
<td>136</td>
<td></td>
<td>&gt; 4</td>
</tr>
<tr>
<td>137</td>
<td></td>
<td>&gt; 4</td>
</tr>
<tr>
<td>138</td>
<td></td>
<td>&gt; 40</td>
</tr>
</tbody>
</table>

$^a$Data were calculated from duplicate experiments by using at least five different inhibitor concentrations.

$^b$For cathepsins L and K, all limits > 40 µM relate to IC$_{50}$ values > 45 µM.

$^c$For cathepsins L and K, all limits > 4 µM relate to IC$_{50}$ values > 5 µM.
In general, the racemic dipeptide nitriles 129–138 showed all fast-binding kinetic behavior and remarkable inhibitory activities toward cathepsin S (40–200 nM). Among them, compounds 129 and 130, containing short aromatic P3 substituents (phenyl and 2-thienyl, respectively), were the most potent cathepsin S inhibitors. The $K_i$ value of the benzoyl substituted dipeptide nitrile 129 on cathepsin S was 40 nM, the corresponding 2-thienyl counterpart exhibited a $K_i$ value of 45 nM. The inhibitory activities of the biaryl derivatives 132 and 135–138 were approximately in the same range ($K_i = 60–94$ nM). However, the $K_i$ value of the 4-(phenyl)-2-thienyl substituted dipeptide nitrile 131 was slightly higher than the $K_i$ values of the biaryl counterparts 132 and 135–138. The fused benzo[b]thiophene-2-yl (133) and thieno[2,3-b]thiophene-2-yl (134) derivatives showed inhibitory activities toward cathepsin S with $K_i$ values of 89 and 100 nM, respectively.

Furthermore, compounds 129 and 130 exhibited an excellent selectivity for cathepsin S over the antitargets, cathepsins L, K and B. The introduction of larger biaryl or fused aromatic P3 substituents impaired the selectivity of the resulting dipeptide nitriles 131–137. The selectivity of the 5-(1H-tetrazole-5-yl)-2-thienyl substituted derivative 138 for cathepsin S over cathepsins L and B was comparable with the selectivities of 129 and 130.

Table 19. Kinetic parameters of compounds 144 and 148.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>cathepsin L</th>
<th>cathepsin S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{on}$ ($10^3$ M$^{-1}$s$^{-1}$)</td>
<td>$k_{off}$ ($10^3$ s$^{-1}$)</td>
</tr>
<tr>
<td>144</td>
<td>n.d.$^d$</td>
<td>n.d.</td>
</tr>
<tr>
<td>148</td>
<td>n.d.$^{d,e}$</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>cmpd</th>
<th>cathepsin K</th>
<th>cathepsin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{on}$ ($10^3$ M$^{-1}$s$^{-1}$)</td>
<td>$k_{off}$ ($10^3$ s$^{-1}$)</td>
</tr>
<tr>
<td>144</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>148</td>
<td>59 ± 2</td>
<td>0.039 ± 0.004</td>
</tr>
</tbody>
</table>

$^d$Data were calculated from duplicate experiments by using at least five different inhibitor concentrations. $^e$Linear regression over 20 min. $^f$Non-linear regression over 80 min. $^g$Not determined. $^h$For [148] = 700 nM, a $k_{obs}$ value could not be obtained by non-linear regression. Therefore, a limit $k_{obs}(1+[S]/K_m)/[I] < 30 \times 10^3$ M$^{-1}$s$^{-1}$ was estimated. $^i$The progress curves were analyzed by linear regression in a time interval between 8 and 16 min. $^j$The progress curves were analyzed by non-linear regression in a time interval of 20 min. $^k$The limit > 40 µM relates to the IC$_{50}$ value > 45 µM.
The enantiopure dipeptide nitrile 144 showed approximately the same selectivity profile as the corresponding racemic compound 129. Hence, it was slightly more potent on cathepsin S (33 versus 44 nM). The azadipeptide nitrile 148 was 60-fold more active on cathepsin S than its carba counterpart 144 (0.55 versus 33 nM), but less selective for cathepsin S over antitargets, cathepsins L, K and B.

While compound 144 was a fast-binding inhibitor, azadipeptide nitrile 148 showed a slow-binding inhibition behavior. Therefore, it was possible to determine $k_{on}$ and $k_{off}$ values for the azadipeptide nitrile 148 and cathepsins S, K and B. For cathepsin L, a limit $k_{obs}(1+[S]/K_m)/[I] \times 10^3 \text{M}^{-1}\text{s}^{-1}$ was estimated. The highest second-order rate constant ($k_{on}$) was obtained for compound 148 and cathepsin S.
2. RESULTS AND DISCUSSION

2.2.2. CONCLUSIONS III

The aim of the study was the exploration of the S3 binding pocket of cathepsin S and the development of selective nitrile inhibitors for this enzyme. A series of dipeptide nitriles was synthesized, in which a large isobutylsulfone moiety was maintained at the P2 position, and a systematic scan for P3 substituents was performed.

For the preparation of inhibitors, a convergent synthetic route was applied in which the P2 building block was separately prepared. Small aromatic, biaryl and annulated aromatic P3 substituents were introduced, and the kinetic properties of the obtained racemic dipeptide nitriles were studied on human cathepsins L, S, K and B. Besides the activity of all tested compounds against cathepsin S, dipeptide nitriles with small aromatic substituents (such as phenyl or 2-thienyl) at the P3 position showed a remarkable selectivity for cathepsin S over cathepsins L, K and B.

Furthermore, the synthetic route to the P2 building block was changed to prevent the racemisation, and the enantiopure benzoyl substituted dipeptide nitrile 144 was obtained as potent and selective cathepsin S inhibitor. The enantiopurity of 144 was shown by chiral HPLC. The aza-analogous counterpart of 144, compound 148, was 60-fold more active on cathepsin S, but less selective for this enzyme over cathepsins L, K and B.

To summarize, the S3 binding pocket of cathepsin S can be addressed with small aromatic as well as with larger biaryl and fused aromatic P3 substituents without significant activity lost of the corresponding inhibitors. However, the introduction of larger biaryl or fused aromatic substituents into the P3 position negatively affected the selectivity of the obtained dipeptide nitriles for cathepsin S. Therefore, the best selectivity profiles were observed for compounds with small aromatic P3 moieties. The enantiopure benzoyl substituted dipeptide nitrile was slightly more active on cathepsin S than its racemic counterpart. The Cα/N replacement resulted in a clearly more potent, but less selective cathepsin S inhibitor.
2.3. DEVELOPMENT OF ‘ACITIVITY-BASED’ PROBES

The knowledge of the cellular and/or plasma activities of cathepsins K and S may be important for the diagnostic of enhanced bone turnover and immune response and could be used for monitoring of the therapeutic success during the treatment of osteoporosis and autoimmune disorders [136], [137], [208], [209]. Furthermore, several cysteine cathepsins, particularly cathepsins L and B, are involved in the tumor pathogenesis and represent possible prognostic marker [210], [211]. In the presented study, it was decided to develop an irreversible, fluorescent ‘activity-based’ probe (ABP) for imaging and quantification particularly of cathepsins K and S, but also cathepsins L and B, by in gel scanning using SDS-PAGE technique. Furthermore, in collaboration with Dr. Reik Löser from the Institute of Radiopharmacy, Helmholtz-Zentrum Dresden-Rossendorf, the tyrosine-derived nitrile inhibitors of cysteine cathepsins were to be fluoroalkylated to prepare ‘cold’ reference compounds of fluorine-18 radiolabeled ‘activity-based’ probes whose kinetic properties had to be studied on cathepsins L, S, K and B.

A convergent synthetic route was chosen for the preparation of the fluorescent ‘activity-based’ probe. First, the vinyl sulfone ‘warhead’ 153 was synthesized as shown in Scheme 30 [212]. Commercially available chloromethyl phenyl sulfide 149 was transformed to the alkyl phosphonate 150 via Arbuzov reaction by heating with triethyl phosphite at 130 °C in a sealed tube. The thioether group of 150 was oxidized with KMnO4 to obtain the sulfone 151. For preparation of the (E)-vinyl sulfone group, Horner-Wadsworth-Emmons reaction was used. In this reaction, compound 151 was coupled with Boc-Gly-H in the presence of NaH to obtain 152. Finally, the Boc protecting group was removed leading to the vinyl sulfone 153.

Scheme 30. Synthesis of the vinyl sulfone 153 as an irreversible, covalent ‘warhead’.
(a) 1. P(OEt)3, 130 °C, sealed tube; (b) KMnO4, AcOH, H2O, rt; (c) Boc-Gly-H, NaH, THF, rt; (d) AcCl, EtOH, AcOEt, rt.
2. RESULTS AND DISCUSSION

With compound 153 in hands, the building block 156 was synthesized as shown in Scheme 31. Boc-protected phenylalanine 154 was coupled via a mixed anhydride with 153 to obtain the vinyl sulfone derivative 155. The Boc protecting group of 155 was cleaved under acidic conditions leading to the building block 156.

![Scheme 31. Synthesis of the building block 156.](image)
(a) 1. ClCO$_2$-i-Bu, NMM, THF, -25 °C, 2. 153, NaOH, H$_2$O, -25 °C to rt; (b) AcCl, EtOH, AcCO$_2$Et, rt, basic extraction.

The pH-dependent GFP-like [213], [214] fluorophore 157 was provided by Ilia V. Yampolsky from the Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow. It was coupled with the building block 156 via a mixed anhydride to obtain the desired ‘activity-based’ probe 168 as shown in Scheme 32.

![Scheme 32. Synthesis of the activity-based probe 158.](image)
(a) 1. ClCO$_2$-i-Bu, NMM, THF, -25 °C, 2. 156, -25 °C to rt.

The fluoroethylated azadipeptide nitrile 159 was synthesized as depicted in Scheme 33.

![Scheme 33. Synthesis of the fluorine-containing dipeptide nitrile 160.](image)
(a) 1. ClCO$_2$-i-Bu, NMM, THF, -25 °C, 2. NH$_2$CH$_2$CN × H$_2$SO$_4$, NaOH, H$_2$O, -25 °C to rt; (b) NaH, 1-bromo-2-fluoroethane, DMF, rt
Cbz-protected tyrosine 26 was reacted via a mixed anhydride with aminoacetonitrile to obtain the dipeptide nitrile 159. The hydroxyl group of 159 was alkylated with 1-bromo-1-fluoroethane in the presence of sodium hydride leading to compound 160. Additionally, the azadipeptide nitrile 38 was fluoroethylated using the same synthetic procedure to obtain the azadipeptide derivative 161 (Scheme 33).

Scheme 33. Synthesis of the fluorine-containing dipeptide nitrile 161.

(a) NaH, 1-bromo-2-fluoroethane, DMF, rt.
2.3.1. KINETIC CHARACTERISATION OF 158 AND 160, 161

The fluorescent ‘activity-based’ probe 158 was tested on human cathepsins L, S, K and B and showed the time-dependent inhibition behavior. In contrast to azadipeptide nitriles, vinyl/allyl sulfones/ketones were described as irreversible, covalent inhibitors of cysteine cathepsins [212], [215], [216]. To determine first-order rate constants (\(k_{\text{obs}}\)) for the irreversible time-dependent inhibition of cathepsins by 158, the progress curves were analyzed by non-linear regression using the equation shown in Figure 41a [216], [217], which is derived from the slow-binding equation (Fig. 23a) for the case, if \(v_s\) tends to zero. Furthermore, the plots of the calculated \(k_{\text{obs}}\) values versus increasing concentrations of 158 showed to be hyperbolic (Fig. 40b) suggesting the enzyme-inhibitor interaction according to the mechanism B (Fig. 25b). Therefore, \(k_{\text{inac}}\) and \(K_i'\) constants could be obtained by non-linear regression of the data pairs \((k_{\text{obs}}, [I])\) using the equation in Figure 41b. The true inhibition constants \((K_i)\) were calculated as described before (Cheng-Prusoff equation, Fig. 23c). The second order rate constants \((k_{2\text{nd}})\) were calculated as quotient of \(k_{\text{inac}}\) divided by \(K_i\) (Fig. 41c). Compounds 160 and 161 were kinetically characterized as described in 2.1.2.

![Figure 40](image-url)
(a) \[ \frac{E}{I} = A\left(1 - e^{-k_{inac}t}\right) + d \]

(b) \[ k_{obs} = \frac{k_{inac}[I]}{\left(K_i' + [I]\right)} \]

(c) \[ k_{2nd} = \frac{k_{inac}}{K_i} \]

**Figure 41.** Slow-binding equation for irreversible inhibition type (a), equation for calculation of \(k_{inac}\) and \(K_i'\) values (b), and the second-order rate constant equation (c).

(a) \(E\) – extinction; \(I\) – fluorescence intensity; \(A = \frac{v_i}{k_{obs}}\) where \(v_i\) – initial reaction rate (M s\(^{-1}\)); \(k_{obs}\) – first-order rate constant (s\(^{-1}\)); \(t\) – time (s); \(d\) – offset; (b) \(k_{obs}\) – first-order rate constant (s\(^{-1}\)); \(k_{inac} = k_{+4}\) – first-order inactivation rate constant (s\(^{-1}\)); \(K_i' = k_{-3}/k_{+3}(1+[S]/K_m)\) – apparent inhibition constant (M); \([I]\) – inhibitor concentration (M); (c) \(K_i\) – true inhibition constant (M); \(k_{2nd}\) – second-order rate constant (M\(^{-1}\)s\(^{-1}\)).

The calculated kinetic parameters are depicted in Table 20. In general, the APBs 158 was active against all tested cathepsins, but it showed a slight preference for cathepsin S (\(k_{2nd} = 18000\) M\(^{-1}\)s\(^{-1}\)) and for cathepsin K (\(k_{2nd} = 4600\) M\(^{-1}\)s\(^{-1}\)). The \(K_i\) values, describing the dissoziation of the non-covalent enzyme-inhibitor complex in the case of the irreversible, covalent inhibition type, were particularly low for cathepsins S (0.11 µM) and K (0.24 µM). The first-order inactivation rate constants were approximately in the same range for the four tested cathepsins (0.0011–0.0031 s\(^{-1}\)).

**Table 20.** Kinetic parameters of compound 158.\(^a\)

<table>
<thead>
<tr>
<th>cmpd</th>
<th>cathepsin L</th>
<th>cathepsin S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_{2nd}) (M(^{-1})s(^{-1}))</td>
<td>(k_{inac}) (s(^{-1}))</td>
</tr>
<tr>
<td>158</td>
<td>2200 ± 180</td>
<td>0.0031 ± 0.0001</td>
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<table>
<thead>
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<th>cmpd</th>
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<th>cathepsin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_{2nd}) (M(^{-1})s(^{-1}))</td>
<td>(k_{inac}) (s(^{-1}))</td>
</tr>
<tr>
<td>158</td>
<td>4600 ± 900</td>
<td>0.0011 ± 0.0001</td>
</tr>
</tbody>
</table>

\(^a\)The progress cuves were analyzed by non-linear regression in a time interval of 30 min.

The fluoroethylated dipeptide nitrile 160 (Table 21), a fast-binding inhibitor, showed only moderate inhibitory activity on cathepsins L, S and K (\(K_i = 390, 1200,\) and \(2100\) nM, respectively). The \(K_i\) value of 160 on the carboxydipeptidase, cathepsin B, was 23 µM.
contrast to 160, the corresponding azadipeptide nitrile 161 exhibited a very strong inhibitory activity toward cathepsins L, S, K and B with $K_i$ values in picomolar range (excepted cathepsin B). In general, the calculated second-order rate constants ($k_{on}$) reflected the low $K_i$ values of 161 on tested cathepsins. The $k_{off}$ values were, as expected, in the same range.

Table 21. Kinetic parameters of compound 160$^a$ and 161$^b$.

<table>
<thead>
<tr>
<th>cmpd</th>
<th>cathepsin L</th>
<th>cathepsin S</th>
<th>cathepsin K</th>
<th>cathepsin B</th>
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<tbody>
<tr>
<td></td>
<td>$k_{on}$ (10$^3$ M$^{-1}$s$^{-1}$)</td>
<td>$k_{off}$ (10$^3$ s$^{-1}$)</td>
<td>$K_i$ (nM)</td>
<td>$k_{on}$ (10$^3$ M$^{-1}$s$^{-1}$)</td>
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<tr>
<td>160</td>
<td>n.d.$^c$</td>
<td>n.d.</td>
<td>390 ± 30</td>
<td>n.d.</td>
</tr>
<tr>
<td>161</td>
<td>930 ± 100$^d$</td>
<td>0.68 ± 0.09</td>
<td>0.73 ± 0.06</td>
<td>560 ± 100$^d$</td>
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</table>

<table>
<thead>
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<th>cmpd</th>
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<th>cathepsin B</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$k_{on}$ (10$^3$ M$^{-1}$s$^{-1}$)</td>
<td>$k_{off}$ (10$^3$ s$^{-1}$)</td>
</tr>
<tr>
<td>160</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>161</td>
<td>210 ± 50</td>
<td>0.036 ± 0.009</td>
</tr>
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</table>

$^a$Linear regression over 20 min. $^b$Non-linear regression over 80 min. $^c$Not determined. $^d$Non-linear regression over 20 min.
2.3.2. SPECTRAL PROPERTIES OF 158 AND IMAGING EXPERIMENT

The absorption and emission spectra of the fluorescent ‘activity-based’ probe 158 were recorded in different solvents to study the spectral properties of this fluorescent compound. The absorption maxima of 158 were approximately in the same range in methanol, water and different aqueous buffer systems, but a slight bathochromic shift was observed in the apolar dichloromethane medium. In contrast to the emission spectra of 158 in the organic solvents such as methanol and dichloromethane, the corresponding emission spectra in water and aqueous buffer systems showed two emission maxima (Fig. 42, Table 22).

![Spectral properties of 158 in different solvents.](image)

**Figure 42.** Spectral properties of 158 in different solvents.
(a) Normalized absorption and emission spectra of compound 158 in different solvents. The compound 158 was used in a concentration of 10 µM. (•) CH$_2$Cl$_2$; (•) EtOH; (•) H$_2$O. (b) Normalized absorption and emission spectra of compound 158 at different pH. (•) H$_2$O; (•) 50 mM sodium phosphate buffer pH 6.5; (•) 100 mM sodium phosphate buffer pH 6.0; (•) 50 mM sodium acetate buffer pH 5.0.

**Table 22.** Absorption and emission maxima of compound 158.

<table>
<thead>
<tr>
<th></th>
<th>CH$_2$Cl$_2$</th>
<th>EtOH</th>
<th>H$_2$O</th>
<th>buffer pH 6.5$^a$</th>
<th>buffer pH 6.0</th>
<th>buffer pH 5.0</th>
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</thead>
<tbody>
<tr>
<td>$\lambda_{ex}$ (nm)</td>
<td>423</td>
<td>415</td>
<td>410</td>
<td>411</td>
<td>411</td>
<td>410</td>
</tr>
<tr>
<td>$\lambda_{em}$ (nm)</td>
<td>480</td>
<td>480</td>
<td>486/528</td>
<td>484/526</td>
<td>484/526</td>
<td>486/528</td>
</tr>
</tbody>
</table>

$^a$For exact buffer composition, see the ‘Experimental Section’.
To prove the concept, compound 158 was incubated with activated cathepsin K. The final concentrations in the incubation medium were 10 µM 158 and 9.2 µg/mL cathepsin K (for details, see the ‘Experimental Section’). The different volumes of the incubation medium as well as two control samples were separated by SDS-PAGE, and the resulting protein bands were analyzed using fluorescence imaging and Coomassie staining (Fig. 43).

The fluorescence analysis showed fluorescent bands of the labeled cathepsin K in the lines III–VI. The fluorescence intensity was dependent on the loaded amounts of the labeled protein and decreased from III to VI. In control experiments (lines I and II) no fluorescence was measured. The fluorescence was excited at 312 nm and imaged with a super sensitive ‘scientific grade’ CCD-camera (equipped with an ethidium bromide filter) from Intas. Although the excitation was not performed in the absorption maximum of 158, it was possible to detect 23 ng of the labeled cathepsin K (VI, Fig 43a). Probably, it will be feasible to image smaller amounts of cysteine cathepsins, if a transilluminator with an excitation wavelength of ~ 400 nm will be used.

For comparative investigations, the same SDS-PAGE was stained with Coomassie brilliant blue. The cathepsin K bands were detected in the lines II–V, but, as expected, not in the control line I. Due to the small amount of cathepsin K, no stained band could be observed in the line VI (Fig. 43b).

Figure 43. SDS-PAGE images. (a) Fluorescence analysis of the with compound 158 labeled cathepsin K ($\lambda_{ex} = 312$ nm, ethidium bromide filter, invert image: white to black); (b) Analysis of cathepsin K bands using Coomassie staining. (I) 130 ng of 158; (II) 184 ng cathepsin K; (III) 184 ng labeled cathepsin K; (IV) 92 ng labelled cathepsin K; (V) 46 ng labelled cathepsin K; (VI) 23 ng labelled cathepsin K; PS – protein standard. The unbound amounts of 158 are not shown.
2.3.3. CONCLUSIONS IV

In the presented study, the fluorescent ‘activity-based’ probe 158, containing a new GFP-like reporter, was synthesized, and its spectral and kinetic properties were studied.

The vinyl sulfone derivative 158 exhibited the time-dependent irreversible kinetic type on cathepsins L, S, K and B according to the two step mechanism of inhibition. In the main, the irreversible, covalent inhibitor 158 was active on all tested cathepsins, but it showed a slight preference for cathepsins S and K.

The absorption maxima of 158 were approximately in the same VIS range in organic solvents and aqueous media. Only a slight bathochromic shift was observed in dichloromethane. In contrast, the emission spectra of 158 were different depending on the solvent, and showed in water and aqueous buffers two emission maxima.

Furthermore, the possibility to image the cysteine cathepsins on SDS-PAGE by compound 158 was proved on example of cathepsin K. The fluorescence analysis of the resulting gel allowed the imaging of fluorescence-labeled cathepsin K. No fluorescence was measured in the control experiments.

In the second project of this study, the fluoroethylated tyrosine-derived dipeptide nitrile 160 as well as its aza-analogue 161 were synthesized as ‘cold’ reference compounds of fluorine-18 radiolabeled ‘activity-based’ probes to study their kinetic properties on cathepsins L, S, K and B. While the fluoroethylated dipeptide nitrile 160, a fast-binding inhibitor, was moderate active toward tested cathepsins, the corresponding azadipeptide nitrile 161 with the slow-binding kinetic behavior was clearly more active.

The preliminary results of the described projects could be used to develop fluorescent and radioactive imaging instruments for cysteine cathepsins in the areas of the Cell and Molecular Biology. Furthermore, the fluorescent ‘activity-based’ probe 158 represents a potential tool for quantification of cathepsins S and K in human (e.g. in blood plasma) to diagnose and monitor osteoporosis and autoimmune disorders.
3. EXPERIMENTAL SECTION
3.1. INHIBITION ASSAYS AND EQUATIONS

3.1.1. CATHEPSIN L INHIBITION ASSAY (I)

Human recombinant cathepsin L (Calbiochem, Darmstadt, Germany) was assayed spectrophotometrically (Cary 50 Bio, Varian) at 405 nm and at 37 °C. The reactions were followed over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. Assay buffer was 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, and 0.01% Brij 35. An enzyme stock solution of 50 µg/mL in 20 mM sodium acetate buffer pH 5.0, 100 mM NaCl, 10 mM trehalose, 1 mM EDTA, and 50% glycerol was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. This enzyme solution was diluted 1:5 with assay buffer containing 5 mM DTT. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-pNa was prepared with DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 100 µM (= 10.0 $K_m$). Assays were performed with a final concentration of 4 ng/mL of cathepsin L. Into a cuvette containing 940 µL assay buffer, inhibitor solution and DMSO in a total volume of 10 µL, and 10 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 40 µL of the cathepsin L solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3. EXPERIMENTAL SECTION

3.1.2. CATHEPSIN L INHIBITION ASSAY (II)

Human isolated cathepsin L (Enzo Life Sciences, Lörrach, Germany) was assayed spectrophotometrically (Cary 50 Bio, Varian) at 405 nm and at 37 °C. The reactions were followed over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. Assay buffer was 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, and 0.01% Brij 35. An enzyme stock solution of 135 µg/mL in 20 mM malonate buffer pH 5.5, 400 mM NaCl, and 1 mM EDTA was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-pNA was prepared with DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 100 µM (= 5.88 \( K_m \)). Assays were performed with a final concentration of 54 ng/mL of cathepsin L. Into a cuvette containing 940 µL assay buffer, inhibitor solution and DMSO in a total volume of 10 µL, and 10 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 40 µL of the cathepsin L solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3.1.3. CATHEPSIN S INHIBITION ASSAY (I)

Cathepsin S (Calbiochem, Darmstadt, Germany) was assayed spectrophotometrically (Cary 50 Bio, Varian) at 405 nm and at 37 °C. The reactions were followed over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. Assay buffer was 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 2 mM EDTA, and 0.01% Triton X-100. An enzyme stock solution of 375 µg/mL in 35 mM potassium phosphate, 35 mM sodium acetate pH 6.5, 2 mM DTT, 2 mM EDTA, and 50% ethylene glycol was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Val-Arg-pNA was prepared with DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 100 µM (= 1.49 $K_m$). Assays were performed with a final concentration of 75 ng/mL of cathepsin S. Into a cuvette containing 960 µL assay buffer, inhibitor solution and DMSO in a total volume of 10 µL, and 10 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 20 µL of the cathepsin S solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3. EXPERIMENTAL SECTION

3.1.4. CATHEPSIN S INHIBITION ASSAY (II)

Cathepsin S (Calbiochem, Darmstadt, Germany) was assayed spectrophotometrically (Cary 50 Bio, Varian) at 405 nm and at 37 °C. The reactions were followed over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. Assay buffer was 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 2 mM EDTA, and 0.01% Triton X-100. An enzyme stock solution of 375 µg/mL in 35 mM potassium phosphate, 35 mM sodium acetate pH 6.5, 2 mM DTT, 2 mM EDTA, and 50% ethylene glycol was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-pNa was used in a final concentration of 100 µM (≈ 0.85 $K_m$). Assays were performed with a final concentration of 75 ng/mL of cathepsin S. Into a cuvette containing 960 µL assay buffer, inhibitor solution and DMSO in a total volume of 10 µL, and 10 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 20 µL of the cathepsin S solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3.1.5. CATHEPSIN K INHIBITION ASSAY (I)

Cathepsin K was assayed fluorimetrically on Monaco Safas spectrofluorometer flx. The wavelength for excitation was 360 nm and for emission 440 nm. The reactions were followed at 25 °C over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. A human recombinant procathepsin K (Calbiochem, Darmstadt, Germany) stock solution of 150 µg/mL in 25 mM Tris buffer pH 8.0, 500 mM NaCl was diluted 1:3.7 with 32.5 mM sodium acetate buffer pH 3.5 and incubated for 3 h at 25 °C to activate procathepsin K to cathepsin K. After incubation, the solution was aliquoted, frozen in liquid nitrogen, and kept at -70 °C. Assay buffer was 100 mM sodium citrate pH 5.0, 100 mM NaCl, 1 mM EDTA, and 0.01% CHAPS. The enzyme solution was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the fluorogenic substrate Z-Leu-Arg-AMC was prepared with DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 40 µM (= 15.4 $K_m$). Assays were performed with a final concentration of 4 ng/mL of cathepsin K. Into a cuvette containing 970 µL assay buffer, inhibitor solution and DMSO in a total volume of 16 µL, and 4 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 10 µL of the cathepsin K solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3.1.6. CATHEPSIN K INHIBITION ASSAY (II)

Cathepsin K was assayed fluorimetrically on Monaco Safas spektrofluorometer flx. The wavelength for excitation was 360 nm and for emission 440 nm. The reactions were followed at 25 °C over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. A human recombinant cathepsin K (Enzo Life Sciences, Lörrach, Germany) stock solution of 23 µg/mL in 50 mM sodium acetate pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT was diluted 1:100 with assay buffer (100 mM sodium citrate pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 °C. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the fluorogenic substrate Z-Leu-Arg-AMC was prepared with DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 40 µM (= 13.3 $K_m$). Assays were performed with a final concentration of 2 ng/mL or 5 ng/mL of cathepsin K. Into a cuvette containing 970 µL or 960 µL assay buffer, inhibitor solution and DMSO in a total volume of 16 µL, and 4 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 10 µL or 20 µL of the cathepsin K solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3.1.7. CATHEPSIN B INHIBITION ASSAY

Human isolated cathepsin B (Calbiochem, Darmstadt, Germany) was assayed spectrophotometrically (Cary 50 Bio, Varian) at 405 nm and at 37 °C. The reactions were followed over 10 min for fast-binding inhibitors and over 80 min for compounds displaying slow-binding behavior, respectively. Assay buffer was 100 mM sodium phosphate buffer pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01% Brij 35. An enzyme stock solution of 1.81 mg/mL in 20 mM sodium acetate buffer pH 5.0, 1 mM EDTA was diluted 1:500 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. Inhibitor stock solutions were prepared in DMSO. A 100 mM stock solution of the chromogenic substrate Z-Arg-ArgpNA was prepared with DMSO. The final concentration of DMSO was 2%, and the final concentration of the substrate was 500 µM (0.45 $K_m$). Assays were performed with a final concentration of 72 ng/mL of cathepsin B. Into a cuvette containing 960 µL assay buffer, inhibitor solution and DMSO in a total volume of 15 µL, and 5 µL of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 20 µL of the cathepsin B solution. Experiments were performed in duplicate with at least five different inhibitor concentrations.
3.1.8. EQUATIONS

Dipeptide nitriles 43, 66, 79, 80, 111, 120, 129–138, 144 and 160 showed a fast-binding inhibition behavior on cathepsins L, S, K and B as reflected by linear progress curves. The apparent inhibition constant \( K_i' \) was determined by non-linear regression using equation
\[
v_s = v_0/(1+[I]/K_i')
\]
where \( v_s \) is the steady-state rate, \( v_0 \) is the rate in the absence of inhibitor, and \([I]\) is the inhibitor concentration. The true inhibition constant \( K_i \) was calculated by correction of \( K_i' \) according to
\[
K_i = K_i'/(1+[S]/K_m)
\]
where \([S]\) is the substrate concentration and \( K_m \) is the Michaelis constant.

Progress curves of the reactions of cysteine proteases in the presence of azadipeptide nitriles 33–38, 42, 45, 46, 50, 51, 62–65, 67, 68, 92, 122, 148, 161 were analyzed by nonlinear regression using slow-binding equation
\[
E/I = A(1-\exp(-k_{obs}t))+d
\]
where \( E/I \) is the extinction/fluorescence intensity, \( v_s \) is the steady-state rate, \( v_i \) is the initial rate, \( k_{obs} \) is the observed first-order rate constant, and \( d \) is the offset. To obtain \( K_i' \), \( v_s \) values as well as the \( v_0 \) value were plotted versus the inhibitor concentration \([I]\), according to \( v_s = v_0/(1+[I]/K_i') \), and \( K_i \) was calculated from equation \( K_i = K_i'/(1+[S]/K_m) \). The apparent second-order rate constant \( k_{on}' \) was obtained by linear regression according to equation \( k_{obs} = k_{on}'[I]+k_{off} \). The true rate constant \( k_{on} \) was calculated by correction of \( k_{on}' \) according to equation \( k_{on} = k_{on}'(1+[S]/K_m) \). The first-order rate constant \( k_{off} \) for the dissociation of the enzyme-inhibitor complex was calculated according to equation \( k_{off} = k_{on}K_i \).

Progress curves of the reactions of cysteine proteases in the presence of the irreversible ‘activity-based’ probe 158 were analyzed by non-linear regression using the slow-binding equation for the irreversible inhibition type
\[
k_{obs} = k_{inac}[I]/(K_i'[1+[I]])
\]
where \( k_{obs} \) is the observed first-order rate constant, and \([I]\) is the inhibitor concentration. The true second-order rate constant \( k_{2nd} \) was calculated according to \( k_{2nd} = k_{inac}/K_i \).
3.2. SPECTRAL PROPERTIES AND IMAGING EXPERIMENT

The absorption spectra were performed on Cary 50 Bio device from Varian. Into a cuvette containing 990 µL solvent (dichloromethane, methanol, water), 10 µL of 122 in DMSO (10 mM) were added, and it was thoroughly mixed. In the case of 158, 10 µL of the compound stock solution in DMSO (1 mM) were added to 990 µL solvent (dichloromethane, methanol, water, cath S buffer pH 6.5, cath L/B buffer pH 6.0, and cath K buffer pH 5.0). The absorption spectra were recorded in a range between 800 and 200 nm after baseline correction. The emission spectra were performed on Monaco Safas spectrophotometer flx in a range between 800 and 200 nm after baseline correction with the same solutions and in the same concentrations of 152 and 158 as in the case of the absorption measurements.

For the imaging experiment, 20 µL of the human recombinant cathepsin K from Enzo 23 µg/mL in 50 mM sodium acetate pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT was added to 29 µL of 100 mM sodium citrate buffer pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS, 5 mM DTT, and it was activated for 40 min at 37 °C. 0.5 µL of DMSO and 0.5 µL of 158 in DMSO (1 mM) were added to the activated cathepsin K, and the resulting solution (9.2 µg/mL cathepsin K, 10 µM ABP 158) was incubated for 40 min at 37 °C. 20 µL, 10 µL, 5 µL, and 2.5 µL of the incubation medium were treated with the reducing (supplemented with 2% (v/v) 2-mercaptoethanol) SDS-loading buffer, heated at 90 °C for 5 min, and separated by SDS/PAGE on the 14% (w/v) polyacrylamide gel. In the control experiment, 20 µL of the activated cathepsin K (9.2 µg/mL) in the absence of 158, and 20 µL of 158 (10 µM in 100 mM sodium citrate buffer pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS, 5 mM DTT, 1% DMSO) were used. The fluorescence analysis of the resulting gel was performed with the Gel iX Imager from Intas, equipped with UV-transilluminator (312 nm), super sensitive ‘scientific grade’ CCD-camera, and an ethidium bromide filter. The Coomassie staining was carried out with Page Blue protein staining solution from Fermentas.
3. EXPERIMENTAL SECTION

3.3. PREPARATION OF COMPOUNDS

3.3.1. GENERAL METHODS AND MATERIALS

Melting points were determined on a Büchi 510 oil bath apparatus, and are uncorrected. Thin layer chromatography was performed on Merck aluminium sheets. Preparative column chromatography was performed on silica gel 60, 0.060–0.200 mm. $^{13}$C NMR (125 MHz) and $^1$H NMR (500 MHz) spectra were recorded on a Bruker Avance DRX 500 spectrometer. Elemental analyses were performed with a Vario EL apparatus. LC-DAD chromatograms and ESI-MS spectra were recorded on an Agilent 1100 HPLC system with Applied Biosystems API-2000 mass spectrometer. The (EI) mass spectra were obtained on A.E.I. MS-50 spectrometer. For compound 158, the MS (ESI) spectra were performed with Bruker Daltonics micrOTOF-Q spectrometer. The chiral analytical HPLC was performed on a Jasco 200 device using LiChroCART® 250-4 ChiraDex® (5 µm) column. Optical rotation was determined on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer. Amino acid derivatives were obtained from Bachem (Bubendorf, Switzerland), Acros (Geel, Belgium) and Aldrich (Steinheim, Germany).
3.3.2. PREPARATION OF 33

![Chemical structure](image)

**N-(Benzyloxycarbonyl)glycyl-methylazaalanine-nitrile.** Compound 21 (1.40 g, 6.69 mmol) was dissolved in dry THF (15 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.74 mL, 6.73 mmol) and isobutyl chloroformate (0.88 mL, 6.75 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (4.44 g, 33.4 mmol) was suspended in H₂O (1 mL), and 5 N NaOH (13.4 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1 × 40, 3 × 10 mL). The combined organic layers were washed with H₂O (15 mL), sat. NaHCO₃ (2 × 15 mL), H₂O (15 mL), and brine (15 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was recrystallized from ethyl acetate/petroleum ether to obtain 27 as a white solid (0.39 g, 23%). Sodium acetate (0.28 g, 3.41 mmol) and cyanogen bromide (0.25 g, 2.36 mmol) were added to a solution of 27 (0.30 g, 1.19 mmol) in MeOH (10 mL). The mixture was stirred at room temperature for 18 h, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 33 as a white solid (0.22 g, 67% from 27). mp 82–86 °C; ¹H NMR (500 MHz, CDCl₃) δ 3.13, 3.20 (2 × s, 2 × 3H, N(CH₃)CN, CONCH₃), 4.12 (dd, ²J = 17.8 Hz, ³J = 3.3 Hz, 1H, NHCH/HCO), 4.23 (dd, ²J = 18.0 Hz, ³J = 5.7 Hz, 1H, NHCH/HCO), 5.11 (s, 2H, CH₂O), 5.46 (bs, 1H, NHCH₂CO), 7.28–7.35 (m, 5H, H_arom); ¹³C NMR (125 MHz, CDCl₃) δ 30.39 (N(CH₃)CN), 40.85 (CONCH₃), 42.51 (NHCH₂CO), 67.09 (CH₂O), 112.94 (CN), 128.04 (C-2’, C-6’), 128.17 (C-4’), 128.50 (C-3’, C-5’), 136.14 (C-1’), 156.24 (OCON), 169.98 (NHCH₂CO); FTIR (KBr, cm⁻¹) 2223 (C≡N); Anal. C₁₃H₁₆N₄O₃ (276.29 g/mol) calcd C 56.51, H 5.84, N 20.28; found C 56.41, H 5.81, N 19.03.
3. EXPERIMENTAL SECTION

3.3.3. PREPARATION OF 34

\[ \text{N-}(\text{Benzyloxycarbonyl})\text{alanil-methylazaalanine-nitrile.} \]

Compound 22 (1.50 g, 6.72 mmol) was dissolved in dry THF (15 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.74 mL, 6.73 mmol) and isobutyl chloroformate (0.88 mL, 6.75 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (4.44 g, 33.4 mmol) was suspended in H\textsubscript{2}O (1 mL), and 5 N NaOH (13.4 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1 × 40, 3 × 10 mL). The combined organic layers were washed with H\textsubscript{2}O (15 mL), sat. NaHCO\textsubscript{3} (2 × 15 mL), H\textsubscript{2}O (15 mL), and brine (15 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and evaporated. The crude product was purified using ethyl acetate/petroleum ether (2:1) to obtain 28 as a colourless oil (1.18 g, 66%). Sodium acetate (1.20 g, 14.6 mmol) and cyanogen bromide (0.65 g, 6.14 mmol) were added to a solution of 28 (1.10 g, 4.15 mmol) in MeOH (15 mL). The mixture was stirred at room temperature for 20 h, and the solvent was removed under reduced pressure. The residue was suspended in H\textsubscript{2}O (10 mL), a pH of 1–2 was adjusted (10% KHSO\textsubscript{4}), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H\textsubscript{2}O (10 mL), sat. NaHCO\textsubscript{3} (2 × 10 mL), H\textsubscript{2}O (10 mL), and brine (10 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and removed in vacuo. The semisolid residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:2) as eluent to obtain 34 as a white solid (0.62 g, 51% from 28). mp 155–158 °C; [\(\alpha\)]\textsubscript{20}\textdegree = +8.0 (c = 0.75, CHCl\textsubscript{3}); \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) mixture rotamers (only the data of the major rotational isomer are noted) \(\delta\) 1.40 (d, \(^3J = 7.3\) Hz, 3H, CH\textsubscript{3}), 3.19, 3.22 (2 × s, 2 × 3H, N(CH\textsubscript{3})CN, CONCH\textsubscript{3}), 4.78–4.83 (m, 1H, NHCHCO), 5.02 (d, \(^2J = 12.6\) Hz, 1H, CHHCO), 5.10 (d, \(^2J = 12.3\) Hz, 1H, CHHO), 5.37 (d, \(^3J = 7.3\) Hz, 1H, NHCHCO), 7.28–7.36 (m, 5H, H\textsubscript{arom}); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \(\delta\) 18.24 (CH\textsubscript{3}), 30.50 (N(CH\textsubscript{3})CN), 40.97 (CONCH\textsubscript{3}), 46.99 (NHCHCO), 66.97 (CH\textsubscript{2}O), 113.55 (CN), 127.97 (C-2\textsuperscript{`}, C-6\textsuperscript{`}), 128.20 (C-4\textsuperscript{`}), 128.53 (C-3\textsuperscript{`}, C-5\textsuperscript{`}), 136.06 (C-1\textsuperscript{`}), 155.89 (OCON), 174.54 (NHCHCO); FTIR (KBr, cm\textsuperscript{-1}) 2226 (C≡N); Anal. C\textsubscript{14}H\textsubscript{18}N\textsubscript{4}O\textsubscript{3} (290.32 g/mol) calcd C 57.92, H 6.25, N 19.30; found C 57.51, H 6.24, N 18.97.
3.3.4. PREPARATION OF 35

*N-(Benzyloxycarbonyl)valyl-methylazaalanine-nitrile*. Compound 23 (1.68 g, 6.69 mmol) was dissolved in dry THF (15 mL) and cooled to -25 °C. To the stirred solution, *N*-methylmorpholine (0.74 mL, 6.73 mmol) and isobutyl chloroformate (0.88 mL, 6.75 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (4.44 g, 33.4 mmol) was suspended in H₂O (1 mL), and 5 N NaOH (13.4 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of *N*-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1 × 40, 3 × 10 mL). The combined organic layers were washed with H₂O (15 mL), sat. NaHCO₃ (2 × 15 mL), H₂O (15 mL), and brine (15 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (2:1) to obtain 29 as a colourless oil (1.10 g, 56%). Sodium acetate (0.78 g, 9.51 mmol) and cyanogen bromide (0.54 g, 5.10 mmol) were added to a solution of 29 (1.00 g, 3.41 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 12 h, and the solvent was removed under reduced pressure. The residue was suspended in H₂O (10 mL), a pH of 1–2 was adjusted (10% KHSO₄), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (10 mL), sat. NaHCO₃ (2 × 10 mL), H₂O (10 mL), and brine (10 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (2:1) as eluent to obtain 35 as a white solid (0.42 g, 39% from 29). mp 64–66 °C; [α]₂⁰D = +20.0 (c = 1.10, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.94 (d, 3J = 6.9 Hz, 1H, CH₃CHCH₃), 1.02 (d, 3J = 7.0 Hz, 3H, CH₃CHCH₃), 1.97–2.04 (m, 1H, CH₃CHCH₃), 3.20, 3.23 (2 × s, 2 × 3H, N(CH₃)CN, CONCH₃), 4.65–4.68 (m, 1H, NHCHCO), 5.02 (d, 2J = 12.3 Hz, 1H, CHHO), 5.09 (d, 2J = 12.3 Hz, 1H, CHHO), 5.26 (d, 3J = 9.2 Hz, 1H, NHCHCO), 7.30–7.36 (m, 5H, H_arom); ¹³C NMR (125 MHz, CDCl₃) δ 17.59, 19.56, (CH₃CHCH₃, CH₃CHCH₃), 30.26, 31.12, 41.21 (CH₃CHCH₃, N(CH₃)CN, CONCH₃), 55.60 (NHCHCO), 67.09 (CH₂O), 113.75 (CN), 128.01 (C-2’, C-6’), 128.23 (C-4’), 128.55 (C-3’, C-5’), 136.03 (C-1’), 156.49 (OCON), 173.89 (NHCHCO); FTIR (KBr, cm⁻¹) 2224 (C≡N); Anal. C₁₆H₂₂N₄O₃ (318.37 g/mol) calcd C 60.36, H 6.97, N 17.60; found C 60.63, H 7.00, N 16.97.
3.3.5. PREPARATION OF 36

**N-(Benzyloxy carbonyl)isoleucyl-methylazaalanine-nitrile.** Compound 24 (1.20 g, 4.52 mmol) was dissolved in dry THF (15 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.50 mL, 4.55 mmol) and isobutyl chloroformate (0.59 mL, 4.53 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (2.70 g, 20.3 mmol) was suspended in H₂O (1 mL), and 5 N NaOH (8.20 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1 × 40, 3 × 10 mL). The combined organic layers were washed with H₂O (15 mL), sat. NaHCO₃ (2 × 15 mL), H₂O (15 mL), and brine (15 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was purified using ethyl acetate/petroleum ether (2:1) to obtain 30 as a semisolid product. Sodium acetate (0.48 g, 5.85 mmol) and cyanogen bromide (0.34 g, 3.21 mmol) were added to a solution of 30 (0.65 g, 2.11 mmol) in MeOH (15 mL). The mixture was stirred at room temperature for 20 h, and the solvent was removed. The residue was suspended in H₂O (10 mL), a pH of 1–2 was adjusted (10% KHSO₄), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (10 mL), sat. NaHCO₃ (2 × 10 mL), H₂O (10 mL), and brine (10 mL). The solvent was dried (Na₂SO₄) and removed *in vacuo*. The semisolid residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:2) as eluent to obtain 36 as a colorless oil (0.34 g, 23% from 30). [α]²⁰<sub>D</sub> = +12.7 (c = 1.10, CHCl₃); <sup>1</sup>H NMR (500 MHz, CDCl₃) mixture of rotamers (only the data of the major rotational isomer are noted) δ 0.89 (t, <sup>3</sup>J = 7.4 Hz, 3H, CH₂CH₃), 0.98 (d, <sup>3</sup>J = 7.0 Hz, 3H, CHCH₃), 1.10–1.25 (m, 1H, CHCH₃), 1.52–1.77 (m, 2H, CH₂CH₃), 3.20, 3.23 (2 × s, 2 × 3H, N(CH₃)CN, CONCH₃), 4.70 (app. t, <sup>3</sup>J = 8.4 Hz, 1H, NHCHCO), 5.01 (d, <sup>2</sup>J = 12.0 Hz, 1H, CHHO), 5.08 (d, <sup>2</sup>J = 12.0 Hz, 1H, CHHO), 5.24 (d, <sup>3</sup>J = 9.5 Hz, 1H, NHCHCO), 7.28–7.35 (m, 5H, H<sub>arom</sub>); <sup>1</sup>³C NMR (125 MHz, CDCl₃) δ 11.11 (CH₂CH₃), 15.73 (CHCH₃), 24.38 (CH₂CH₃), 30.25, 37.61, 41.22 (N(CH₃)CN, CHCH₃, CONCH₃), 54.77 (NHCHCO) 67.07 (CH₂O), 113.77 (CN), 127.99 (C-2', C-6'), 128.22 (C-4'), 128.53 (C-3', C-5'), 136.02 (C-1'), 156.41 (OCON), 174.08 (NHCHCO); FTIR (KBr, cm⁻¹) 2223 (C≡N); Anal. C₁₇H₂₄N₄O₃ (332.40 g/mol) calcd C 61.43, H 7.28, N 16.86; found C 61.18, H 7.62, N 16.46.
3.3.6. PREPARATION OF 37

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N-(\text{Benzyloxycarbonyl})\text{cyclohexylalanyl-methylazaalanine-nitrile. Compound 25 (1.00 g, 3.27 mmol) was dissolved in dry THF (15 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.36 mL, 3.27 mmol) and isobutyl chloroformate (0.43 mL, 3.30 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (2.20 g, 16.5 mmol) was suspended in H\textsubscript{2}O (1 mL), and 5 N NaOH (6.50 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1 × 40, 3 × 10 mL). The combined organic layers were washed with H\textsubscript{2}O (15 mL), sat. NaHCO\textsubscript{3} (2 × 15 mL), H\textsubscript{2}O (15 mL), and brine (15 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and evaporated. The crude product was purified using ethyl acetate/petroleum ether (2:1) to obtain 31 as a colourless oil (0.70 g, 62%). Sodium acetate (0.44 g, 5.36 mmol) and cyanogen bromide (0.30 g, 2.83 mmol) were added to a solution of 31 (0.63 g, 1.81 mmol) in MeOH (50 mL). The mixture was stirred at room temperature for 12 h, and the solvent was removed under reduced pressure. The residue was suspended in H\textsubscript{2}O (10 mL), a pH of 1–2 was adjusted (10% KHSO\textsubscript{4}), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H\textsubscript{2}O (10 mL), sat. NaHCO\textsubscript{3} (2 × 10 mL), H\textsubscript{2}O (10 mL), and brine (10 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:2) as eluent to obtain 37 as a colorless oil (0.21 g, 31% from 31). [\alpha]\textsuperscript{20}D = +17.0 (c = 1.00, CHCl\textsubscript{3}); \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}\textsubscript{6}) mixture of rotamers (only the data of the major rotational isomer are noted) δ 0.90–1.79 (m, 13H, H\textsubscript{cyclohexane}), 3.08, 3.20 (2 × s, 2 × 3H, N(CH\textsubscript{3})CN, CONCH\textsubscript{3}), 4.61 (bs, 1H, NHCH\textsubscript{2}CO), 4.98–5.04 (m, 2H, CH\textsubscript{2}O), 7.26–7.37 (m, 5H, H\textsubscript{arom}), 7.71 (d, J\textsubscript{3} = 7.6 Hz, 1H, NHCH\textsubscript{2}CO); \textsuperscript{13}C NMR (125 MHz, DMSO-\textit{d}\textsubscript{6}) δ 25.62, 25.85, 26.13 (C\textsubscript{cyclohexane}), 30.49, 31.58, 33.51 (N(CH\textsubscript{3})CN, C\textsubscript{cyclohexane}), 33.65, 38.18 (CONCH\textsubscript{3}, CHCH\textsubscript{2}), 48.92 (NHCH\textsubscript{2}CO) 65.66 (CH\textsubscript{2}O), 114.24 (CN), 127.84 (C-2"", C-6""), 127.96 (C-4""), 128.47 (C-3"", C-5""), 137.03 (C-1""), 156.43 (OCON), 174.44 (NHCH\textsubscript{2}CO); FTIR (KBr, cm\textsuperscript{-1}) 2222 (C≡N); Anal. C\textsubscript{20}H\textsubscript{28}N\textsubscript{4}O\textsubscript{3} (372.46 g/mol) calcd C 64.49, H 7.58, N 15.04; found C 64.81, H 8.14, N 13.94.}
3.3.7. PREPARATION OF 38

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\begin{align*}
\text{N-}(\text{Benzyloxy carbonyl})\text{-tyrosyl-methylazaalanine-nitrile.} \quad & \text{Compound 26 (10.0 g, 31.7 mmol) was dissolved in dry THF (60 mL) and cooled to -25 °C. To the stirred solution, } \nonumber
\text{N-methylmorpholine (3.84 mL, 34.9 mmol) and isobutyl chloroformate (4.54 mL, 34.8 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (8.43 g, 63.4 mmol) was suspended in H}_2\text{O (15 mL), and 10 N NaOH (13.0 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with H}_2\text{O (30 mL), sat. NaHCO}_3 (2 × 30 mL), H}_2\text{O (30 mL), and brine (30 mL). The solvent was dried (Na}_2\text{SO}_4\) and evaporated to obtain 32 as a white solid. Sodium acetate (4.22 g, 51.4 mmol) and cyanogen bromide (4.09 g, 38.6 mmol) were added to a solution of 32 (9.20 g, 25.7 mmol) in MeOH (40 mL). The mixture was stirred at room temperature for 24 h, and the solvent was removed. The residue was suspended in H}_2\text{O (30 mL), a pH of ~2 was adjusted (10% KHSO}_4\), and it was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with H}_2\text{O (30 mL), sat. NaHCO}_3 (30 mL), H}_2\text{O (30 mL), and brine (30 mL). The solvent was dried (Na}_2\text{SO}_4\) and evaporated to obtain in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 38 as a white solid (2.82 g, 23% from 26). mp 158 °C; [\alpha]^{20}_D = +30.0 (c = 1.00, CHCl}_3\); \text{H NMR (500 MHz, DMSO-d}_6\) mixture of rotamers (only the data of the major rotational isomer are noted) \(\delta\) 2.67–2.80 (m, 2H, CHCH}_2\), 3.11, 3.22 (2 × s, 2 × 3H, N(CH}_3\)CN, CONCH}_3\), 4.65 (bs, 1H, NHCHCO), 4.95 (s, 2H, CH}_2\text{O}), 6.67–7.35 (m, 9H, H}_arom\), 7.82 (d, \(3^J = 7.6\) Hz, 1H, NHCHCO), 9.21 (s, 1H, OH); \text{C NMR (125 MHz, DMSO-d}_6\) mixture of rotamers, w = weak (minor rotational isomer), i = intensive (major rotational isomer) \(\delta\) 30.17 (w), 30.55 (i), 35.69 (i), 36.23 (w) (N(CH}_3\)CN, CONCH}_3\), 40.26 (w), 40.45 (i) (CHCH}_2\), 52.93 (w), 53.46 (i) (NHCHCO), 65.58 (CH}_2\text{O), 114.21 (CN), 115.21, 127.69, 127.88, 128.41, 130.11 (i), 130.33, 136.92 (i), 137.04 (w) (C-3’, C-5’, C-2’’, C-6’’, C-4’’, C-3’’, C-5’’, C-2’, C-6’, C-1’, C-1’’), 156.20 (OCON, C-4’), 173.60 (NHCHCO); Anal. C\text{O}_2\text{H}_2\text{N}_4\text{O}_4\) (382.41 g/mol) calcd C 62.82, H 5.80, N 14.65; found C 63.11, H 5.86, N 14.20.} \text{)}
3.3.8. PREPARATION OF 40

\[ N-(\text{Benzyloxy carbonyl})\text{homocycloleucine 1,2-dimethylhydrazide}. \]

Method A, mixed anhydride coupling protocol: Compound 39 (2.00 g, 7.21 mmol) was dissolved in THF (20 mL) and cooled to -25 °C. Triethylamine (4.38 g, 43.3 mmol) and isobutyl chloroformate (1.08 g, 7.91 mmol) were added, and it was stirred for 3 min at -25 °C. 1,2-dimethylhydrazine dihydrochloride (2.40 g, 18.0 mmol) was added. The resulting reaction mixture was stirred for 19 h at room temperature. THF was evaporated under reduced pressure. The residue was extracted with ethyl acetate (3 × 30 mL), washed with sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried over Na₂SO₄ and removed under reduced pressure. The oily reaction mixture was separated by column chromatography using ethyl acetate/petroleum ether 9:1 as eluent to obtain 40 as a colorless oil (0.41 g, 18%).

\[ N-(\text{Benzyloxy carbonyl})\text{homocycloleucine 1,2-dimethylhydrazide}. \]

Method B, EDC/DMAP coupling protocol, rt: Compound 39 (1.00 g, 3.61 mmol) was dissolved in dry THF (10 mL). DMAP (0.02 g, 0.16 mmol) and EDC (0.62 g, 3.99 mmol) were added, and the solution was stirred for 10 min at room temperature. 1,2-Dimethylhydrazine dihydrochloride (0.96 g, 7.22 mmol) was suspended in dry THF (10 mL) and treated with triethylamine (1.46 g, 14.4 mmol). The resulting reaction mixtures were combined and stirred for 24 h at rt. The solvent was evaporated under reduced pressure. The residue was treated with H₂O and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO₃ (2 × 30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried over Na₂SO₄ and removed under reduced pressure. The crude oily product was purified by column chromatography using CH₂Cl₂/MeOH (19:1) as eluent to obtain 40 as a colorless oil (0.41 g, 36%).

\[ N-(\text{Benzyloxy carbonyl})\text{homocycloleucine 1,2-dimethylhydrazide}. \]

Method C, EDC/DMAP coupling protocol, Δ: Compound 39 (1.00 g, 3.61 mmol) was dissolved in dry THF (10 mL). DMAP (0.02 g, 0.16 mmol) and EDC (0.62 g, 3.99 mmol) were added, and the solution was stirred for 10 min at room temperature. 1,2-Dimethylhydrazine dihydrochloride (0.96 g, 7.22 mmol) was suspended in dry THF (10 mL) and treated with triethylamine (1.46 g, 14.4 mmol). The resulting reaction mixtures were combined and heated to reflux for 24 h.
The solvent was evaporated under reduced pressure. The residue was treated with H$_2$O and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO$_3$ (2 × 30 mL), H$_2$O (30 mL), and brine (30 mL). The solvent was dried over Na$_2$SO$_4$ and removed under reduced pressure. The crude oily product was purified by column chromatography using CH$_2$Cl$_2$/MeOH (19:1) as eluent to obtain 40 as a colorless oil (0.45 g, 39%).

*N-(Benzyloxy carbonyl)homocycloleucine 1,2-dimethylhydrazide.* Method D, HBTU coupling protocol (in the presence of HOBt): Compound 39 (1.00 g, 3.61 mmol) was dissolved in dry THF (10 mL). HOBt × H$_2$O (0.24 g, 1.78 mmol) and HBTU (1.51 g, 3.98 mmol) were added, and the solution was stirred for 10 min at room temperature. 1,2-Dimethylhydrazine dihydrochloride (0.96 g, 7.22 mmol) was suspended in dry THF (10 mL) and treated with triethylamine (1.46 g, 14.4 mmol). The resulting reaction mixtures were combined and stirred for 3 d at rt. The solvent was evaporated under reduced pressure. The residue was treated with H$_2$O and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO$_3$ (2 × 30 mL), H$_2$O (30 mL), and brine (30 mL). The solvent was dried over Na$_2$SO$_4$ and removed under reduced pressure. The crude oily product was purified by column chromatography using CH$_2$Cl$_2$/MeOH (19:1) and additionally ethyl acetate/petroleum ether (2:1) as eluents to obtain 40 as a colorless oil (0.31 g, 27%).

$^1$H NMR (500 MHz, DMSO-$_d_6$) $\delta$ 1.13–1.20 (m, 1H, H$_{cyclohexane}$), 1.41–1.51 (m, 5H, H$_{cyclohexane}$), 1.66–1.72 (m, 2H, H$_{cyclohexane}$), 2.11 (bs, 2H, H$_{cyclohexane}$), 2.30 (d, $^3J$ = 5.7 Hz, 3H, NHC$_3$), 2.90 (s, 3H, CONCH$_3$), 4.97 (s, 2H, CH$_2$O), 7.27–7.37 (m, 5H, H$_{arom}$); $^{13}$C NMR (125 MHz, DMSO-$_d_6$) $\delta$ 21.28 (C-3’, C-5’), 25.21 (C-4’), 31.92 (C-2’, C-6’), 34.97 (NHCH$_3$), 58.63 (C-1’), 65.03 (CH$_2$O), 127.80 (C-2’’, C-6’’), 127.86 (C-4’’), 128.36 (C-3’’, C-5’’), 137.36 (C-1’), 154.64 (OCO); MS(ESI) $m/z$ = 320.2 ([M + H]$^+$).


3.3.9. PREPARATION OF 41

![Chemical Structure]

Isobutyl 1,2-dimethylhydrazinecarboxylate. The oily reaction mixture (for reaction conditions, see 3.3.8., method A) was separated by column chromatography using ethyl acetate/petroleum ether (9:1) as eluent to obtain a colorless oil (0.50 g) containing compound 41 contaminated with N-(Benzyloxycarbonyl)homocycloleucine (compound 40, see 3.3.8.). Using $^1$H NMR spectrum, the yield of compound 41 was calculated to 0.32 g (28%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 0.89 (app. d, $^3$J = 6.6 Hz, 6H, CH(CH$_3$)$_2$), 1.86 (sept, $^3$J = 6.7 Hz, 1H, CH(CH$_3$)$_2$), 2.43 (s, 3H, NHCH$_3$), 2.93 (s, 3H, CONCH$_3$), 3.78 (d, $^3$J = 6.6 Hz, 2H, CHCH$_2$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 18.98 (CH(CH$_3$)$_2$), 27.68 (CH(CH$_3$)$_2$), 35.49, 35.59 (NCH$_3$), 70.93 (CH$_2$O), 156.16 (OCO); MS(El) m/z = 160.1 (M$^{++}$).
3.3.10. PREPARATION OF 42

*N-(Benzylxoycarbonyl)*homocycloleucyl-methylazaalanine-nitrile. Compound 40 (0.44 g, 1.38 mmol) was dissolved in MeOH (10 mL) and treated with NaOAc (0.31 g, 3.78 mmol) and BrCN (0.30 g, 2.83 mmol). The resulting reaction mixture was stirred for 18 h at room temperature. The solvent was evaporated under reduced pressure. The residue was suspended in H₂O (10 mL), a pH of 1–2 was adjusted (10% KHSO₄), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (10 mL), sat. NaHCO₃ (2 × 10 mL), H₂O (10 mL), and brine (10 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 42 as a colorless oil (0.24 g, 50%). ¹H NMR (500 MHz, DMSO- d₆) δ 1.16–1.25 (m, 1H, Hcyclohexane), 1.47 (bs, 5H, Hcyclohexane), 1.63–1.69 (m, 2H, Hcyclohexane), 1.98–2.01 (m, 2H, Hcyclohexane), 2.92 (s, 3H, N(CH₃)CN), 3.07 (s, 3H, CONCH₃), 4.97 (CH₂O), 7.29–7.36 (m, 5H, Harom); ¹³C NMR (125 MHz, DMSO-d₆) δ 21.12 (C-3’, C-5’), 24.92 (C-4’), 31.64 (C-2’, C-6’), 40.41 (CONCH₃), 58.45 (C-1’), 65.69 (CH₂O), 115.02 (CN), 128.08 (C-2’’, C-6’’), 128.28 (C-4’’), 128.48 (C-3’’, C-5’’), 137.00 (C-1’’), 154.68 (OCO), 173.58 (NHCOC); Anal. C₁₈H₂₄N₄O₃ (344.41 g/mol) calcd C 62.77, H 7.02, N 16.27; found C 62.70, H 7.17, N 15.49.
3.3.11. PREPARATION OF 43

*N-(Benzyloxy carbonyl)homocycloleucyl-glycine-nitrile.* Compound 39 (1.00 g, 3.61 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. To the stirred solution, triethylamine (1.09 g, 10.8 mmol) and isobutyl chloroformate (0.54 g, 3.95 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.64 g, 10.6 mmol) was added to the reaction mixture when the precipitation of triethylamine hydrochloride occurred (ca. 5 min). It was allowed to warm to room temperature within 30 min, and stirred for additional overnight. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL), H₂O (30 mL), sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and evaporated. The product mixture was separated by column chromatography using ethyl acetate/petroleum ether (1:1) as eluent to obtain 43 as a white solid (0.60 g, 53%).

mp 110–112 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 1.15–1.25 (m, 1H, Hcyclohexane), 1.45–1.51 (m, 5H, Hcyclohexane), 1.63–1.69 (m, 2H, Hcyclohexane), 1.89–1.92 (m, 2H, Hcyclohexane), 4.03 (d, 3J = 5.1 Hz, 2H, NHCH₂CN), 5.00 (s, 2H, CH₂O), 7.27–7.36 (m, 6H, OCONH/Harom), 8.20 (bs, 1H, NHCH₂CN); ¹³C NMR (125 MHz, DMSO-d₆) δ 21.03 (C-3’, C-5’), 25.03 (C-4’), 27.76 (NHCH₂CN), 31.81 (C-2’, C-6’), 58.90 (C-1’), 65.28 (CH₂O), 117.82 (CN), 127.74 (C-2’’, C-6’’), 127.84 (C-4’’), 128.43 (C-3’’, C-5’’), 137.20 (C-1’’), 154.86 (OCO), 175.20 (NHCO); Anal. C₁₇H₂₁N₃O₃ (315.37 g/mol) calcd C 64.74, H 6.71, N 13.32; found C 64.76, H 6.72, N 12.81.
3. EXPERIMENTAL SECTION

3.3.12. PREPARATION OF 44

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\begin{align*}
\text{N-} & (\text{Isobutylloxycarbonyl})\text{aminoacetonitrile.} \text{ The product mixture (for reaction conditions, see 3.3.11.)} \text{ was separated by column chromatography using ethyl acetate/petroleum ether (1:1) as eluent to obtain 44 as a colorless oil (0.20 g, 35%).} \\
& \text{\textsuperscript{1}H NMR (500 MHz, DMSO-}d_6\text{) } \delta \\
& 0.87 \text{ (app. d, } \text{J} =6.6 \text{ Hz, } 6\text{H, CH(CH}_3\text{)}_2) , 1.80\text{--1.88 (m, } 1\text{H, CH(CH}_3\text{)}_2) , 3.79 \text{ (d, } \text{J} =6.7 \text{ Hz, } 2\text{H, CH}_2\text{O), 4.05 (d, } \text{J} =6.0 \text{ Hz, } 2\text{H, NHCH}_2\text{CN), 7.84 (bs, NHCH}_2\text{CN); } \text{\textsuperscript{13}C NMR (125 MHz, DMSO-}d_6\text{) } \delta \\
& 19.64 \text{ (CH(CH}_3\text{)}_2) , 28.40 \text{ (CH(CH}_3\text{)}_2) , 29.90 \text{ (NHCH}_2\text{CN), 71.43 (CH}_2\text{O), 118.76 (CN), 157.16 (OCO); MS(ESI) } m/z \text{ (neg.) = 155.1 ([M - H])}. 
\end{align*}
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3.3.13. PREPARATION OF 48

N-(Benzylcarbamoyl)leucine tert-butyl ester. The hydrochloride salt of 47 (2.50 g, 11.2 mmol) was dissolved in H$_2$O (10 mL) and treated with 1 N NaOH solution (13.0 mL). The precipitated free base was extracted with ethyl acetate (3 ¥ 20 mL). The combined organic layers were washed with H$_2$O (10 mL) and brine (10 mL), and dried over Na$_2$SO$_4$. The solvent was removed in vacuo to obtain leucine tert-butyl ester 47 as a colorless oily residue. This residue was dissolved in dry THF (40 mL) and treated with CDI (2.00 g, 12.3 mmol). The resulting solution was heated to reflux for 2 h. The reaction mixture was further treated with benzylamine (2.01 mL, 18.4 mmol) and heated to reflux for 18 h. After evaporation of the solvent, the residue was suspended in H$_2$O. The resulting aqueous suspension was adjusted with 10% KHSO$_4$ to pH ~2 and extracted with ethyl acetate (3 ¥ 20 mL). The combined organic layers were washed with H$_2$O (20 mL), 10% KHSO$_4$ (20 mL), H$_2$O (20 mL), and brine (20 mL). The solvent was dried (Na$_2$SO$_4$) and removed in vacuo. The solid product was purified by column chromatography using ethyl acetate/petroleum ether (1:1) as eluent to obtain 48 as a white solid (3.25 g, 91%). mp 69 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 0.86 (d, $^3$J = 6.3 Hz, 3H, CH$_3$CHCH$_3$), 0.89 (d, $^3$J = 7.0 Hz, 3H, CH$_3$CHCH$_3$), 1.39 (s, 9H, C(CH$_3$)$_3$), 1.41–1.44 (m, 2H, CHCH$_2$), 1.59–1.68 (m, 1H, CH$_3$CHCH$_3$), 4.05–4.09 (m, 1H, NHCHO), 4.16–4.24 (m, 2H, CH$_2$NH), 6.15 (d, $^3$J = 8.5 Hz, 1H, NHCHO), 6.40 (t, $^3$J = 6.0 Hz, 1H, CH$_2$NH), 7.19–7.31 (m, 5H, H$_{arom}$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 21.85, 22.81, 24.49 (CH$_3$CHCH$_3$, CH$_3$CHCH$_3$, CH$_3$CHCH$_3$), 27.78 (C(CH$_3$)$_3$), 41.26, 42.94 (CHCH$_2$, CH$_2$NH), 51.86 (NHCHO), 80.27 (C(CH$_3$)$_3$), 126.69 (C-4’), 127.06 (C-2’, C-6’), 128.29 (C-3’, C-5’), 140.83 (C-1’), 157.77 (NHCONH), 173.07 (NHCHO); Anal. C$_{18}$H$_{28}$N$_2$O$_3$ (320.43 g/mol) calcd C 67.47, H 8.81, N 8.74; found C 67.59, H 9.12, N 8.54.
3.3.14. PREPARATION OF 49

*N-(Phenylcarbamoyl)leucine tert-butyl ester.* The hydrochloride salt of 47 (2.50 g, 11.2 mmol) was dissolved in H₂O (10 mL) and treated with 1 N NaOH solution (13.0 mL). The precipitated free base was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (10 mL) and brine (10 mL), and dried over Na₂SO₄. The solvent was removed *in vacuo* to obtain leucine tert-butyl ester 47 as a colorless oily residue. This residue was dissolved in dry THF (40 mL) and treated with CDI (2.00 g, 12.3 mmol). The resulting solution was heated to reflux for 2 h. The solvent was removed *in vacuo,* and the obtained residue was treated with dry MeCN (40 mL) and DIPEA (3.05 mL, 17.8 mmol). Aniline (1.67 mL, 18.3 mmol) was further added, and the reaction mixture was heated to reflux for 39 h. After evaporation of the solvent, the residue was suspended in H₂O. The resulting aqueous suspension was adjusted to pH ~2 with 10% KHSO₄ and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), 10% KHSO₄ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and removed *in vacuo.* The oily product was purified by column chromatography using ethyl acetate/petroleum ether (1:2) as eluent to obtain 49 as a white solid (1.29 g, 38%). mp 106–108 °C; ¹H NMR (500 MHz, DMSO- d₆) δ 0.89 (d, ³J = 6.6 Hz, 3H, CH₃CHCH₃), 0.92 (d, ³J = 6.6 Hz, 3H, CH₃CHCH₃), 1.41 (s, 9H, C(CH₃)₃), 1.44–1.50 (m, 2H, CH₂CH₂), 1.61–1.71 (m, 1H, CH₂CHCH₂), 4.10–4.15 (m, 1H, NHCHCO), 6.35 (d, ³J = 8.2 Hz, 1H, NHCHCO), 6.78–7.37 (m, 5H, H arom), 8.51 (s, 1H, C-1’NH); ¹³C NMR (125 MHz, DMSO- d₆) δ 21.84, 22.77, 24.53 (CH₃CHCH₃, CH₂CHCH₃, CH₂CHCH₃), 27.77 (C(CH₃)₃), 41.13 (CHCH₂), 51.50 (NHCHCO), 80.62 (C(CH₃)₃), 117.72 (C-2’, C-6’), 121.35 (C-4’), 128.78 (C-3’, C-5’), 140.25 (C-1’), 154.87 (NHCONH), 172.69 (NHCHCO); Anal. C₁₇H₂₆N₂O₃ (306.40 g/mol) calcd C 66.64, H 8.55, N 9.14; found C 66.23, H 8.91, N 8.78.
3.3.15. PREPARATION OF 50

*N-(Benzylcarbamoyl)leucyl-methylazaalanine-nitrile.* Compound 48 (2.00 g, 6.24 mmol) was dissolved in CH₂Cl₂ (40 mL) and treated with TFA (20 mL). The resulting solution was stirred for 3 h at room temperature. After evaporation of the solvent, the oily residue was suspended in H₂O. The resulting aqueous suspension was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (2 × 30 mL) and brine (30 mL). The solvent was dried (Na₂SO₄) and removed under reduced pressure. The crude solid product was recrystallized from ethyl acetate/n-hexane to obtain *N-(benzylcarbamoyl)*-leucine (0.52 g, 32%). *N-(Benzylcarbamoyl)leucine* (0.48 g, 1.82 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. To the stirred solution, *N*-methylmorpholine (0.22 mL, 2.00 mmol) and isobutyl chloroformate (0.26 mL, 1.99 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (1.21 g, 9.10 mmol) was suspended in H₂O (1 mL), and 5 N NaOH (4.36 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of *N*-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with sat. NaHCO₃ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and evaporated. The obtained oily residue was purified by column chromatography on silica gel using ethyl acetate as eluent to obtain *N-(benzylcarbamoyl)*-leucine 1,2-dimethylhydrazide. Sodium acetate (0.11 g, 1.34 mmol) and cyanogen bromide (0.07 g, 0.66 mmol) were added to a solution of *N-(benzylcarbamoyl)*-leucine 1,2-dimethylhydrazide (0.18 g, 0.59 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 48 h, and the solvent was removed under reduced pressure. The residue was suspended in H₂O (10 mL), a pH of ~2 was adjusted (10% KHSO₄), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), sat. NaHCO₃ (2 × 20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 50 as a white solid (0.15 g, 25% from *N-(benzylcarbamoyl)*-leucine). mp 116–118 °C; [α]20D = +2.8 (c = 0.58, CHCl₃); ¹H NMR (500 MHz, DMSO-d₆) mixture of rotamers (only the data of the major rotational isomer are noted) δ 0.90–0.93 (m, 6H, CH(CH₃)₂), 1.28–1.43
3. EXPERIMENTAL SECTION

(m, 2H, CHCH₂), 1.68 (bs, 1H, CH(CH₃)₂), 3.08, 3.22 (2 × s, 2 × 3H, N(CH₃)CN, CONCH₃), 4.19 (d, 2H, J = 5.7 Hz, 2H, CH₂NH), 4.74–4.77 (m, 1H, NHCHCO), 6.30 (d, J = 8.6 Hz, 1H, NHCHCO), 6.42 (t, J = 5.7 Hz, 1H, CH₂NH), 7.19–7.31 (m, 5H, H arom); ¹³C NMR (125 MHz, DMSO-d₆) δ 21.44, 23.31, 24.50 (CH₃CHCH₃, CH₂CHCH₃, CH₃CHCH₃), 30.48 (N(CH₃)CN), CONCH₃ obscured by the DMSO signal, 41.00, 42.97 (CHCH₂, CH₂NH), 47.98 (NHCHCO), 114.30 (CN), 126.73 (C-4'), 127.08 (C-2', C-6'), 128.35 (C-3', C-5'), 140.68 (C-1'), 158.01 (NHCONH), 175.32 (NHCHCO); FTIR (KBr, cm⁻¹) 2220 (C≡N); Anal. C₁₇H₂₅N₅O₂ (331.41 g/mol) calcd C 61.61, H 7.60, N 21.13; found C 61.44, H 7.57, N 20.40; LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 200.0–300.0 nm) tᵣ = 15.64, 98% purity, m/z = 332.2 ([M + H]+).
3.3.16. PREPARATION OF 51

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\text{N-(Phenylcarbamoyl)leucyl-methylazaalanine-nitrile.}
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Compound 49 (1.23 g, 4.01 mmol) was dissolved in CH\(_2\)Cl\(_2\) (40 mL) and treated with TFA (20 mL). The resulting solution was stirred for 3 h at room temperature. After evaporation of the solvent, the oily residue was suspended in H\(_2\)O. The aqueous suspension was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H\(_2\)O (20 mL), 10% KHSO\(_4\) (20 mL), H\(_2\)O (20 mL), and brine (20 mL). The solvent was dried (Na\(_2\)SO\(_4\)) and removed under reduced pressure. The solid product was recrystallized from ethyl acetate/n-hexane to obtain N-(phenylcarbamoyl)leucine (0.50 g, 50%). N-(Phenylcarbamoyl)leucine (0.50 g, 2.00 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.24 mL, 2.18 mmol) and isobutyl chloroformate (0.29 mL, 2.22 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (1.47 g, 11.1 mmol) was suspended in H\(_2\)O (1 mL), and 5 N NaOH (5.33 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H\(_2\)O (20 mL), sat. NaHCO\(_3\) (20 mL), H\(_2\)O (20 mL), and brine (20 mL). The solvent was dried (Na\(_2\)SO\(_4\)) and evaporated. The obtained oily residue was purified by column chromatography on silica gel using ethyl acetate as eluent to obtain N-(phenylcarbamoyl)leucine 1,2-dimethylhydrazide. Sodium acetate (0.07 g, 0.85 mmol) and cyanogen bromide (0.04 g, 0.38 mmol) were added to a solution of N-(phenylcarbamoyl)leucine 1,2-dimethylhydrazide (0.11 g, 0.38 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 48 h, and the solvent was removed under reduced pressure. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 51 as a white solid (0.18 g, 28% from N-(phenylcarbamoyl)leucine). mp 103–105 °C; \([\alpha]^{20}_D = +29.5\) (c = 1.23, CHCl\(_3\)); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) mixture of rotamers (only the data of the major rotational isomer are noted) \(\delta 0.93–0.97\) (m, 6H, CH(C\(_\text{H}_3\))\(_2\)), 1.35–1.51 (m, 2H, CHCH\(_2\)), 1.72 (bs, 1H, CH(CH\(_3\))\(_2\)), 3.10, 3.26 (2 × s, 2 × 3H, N(CH\(_3\))CN, CONCH\(_3\)), 4.79–4.82 (m, 1H, NHCHCO), 6.48 (d, \(^3\)J = 7.9 Hz, 1H, NHCHCO), 6.88–7.36 (m, 5H, H\(_{\text{arom}}\)), 8.53 (s, 1H, C-1′NH); \(^13\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta 21.43, 23.30, 24.55\).
3. EXPERIMENTAL SECTION

(CH₂CH₂, CH₃CH₂, CH₃CH₂), 30.53 (N(CH₃)CN), CONCH₃ obscured by the DMSO signal, 41.02 (CH₂), 47.78 (NHCHCO), 114.24 (CN), 117.80 (C-2', C-6'), 121.49 (C-4'), 128.81 (C-3', C-5'), 140.09 (C-1'), 155.20 (NHCONH), 174.88 (NHCHCO); FTIR (KBr, cm⁻¹) 2224 (C≡N); Anal. C₁₆H₂₃N₅O₂ (317.39 g/mol) calcd C 60.55, H 7.30, N 22.07; found C 60.11, H 7.36, N 21.15; LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) tᵣ = 16.24, 94% purity, m/z = 318.3 ([M + H]⁺).
3.3.17. PREPARATION OF 52

\[ \text{N-(4-Cyanobenzylcarbamoyl)leucine tert-butyl ester.} \]

The hydrochloride salt of 47 (5.00 g, 22.3 mmol) was dissolved in H\textsubscript{2}O (10 mL) and treated with 1 N NaOH solution (24.0 mL). The precipitated free base was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H\textsubscript{2}O (10 mL) and brine (20 mL), and dried over Na\textsubscript{2}SO\textsubscript{4}. The solvent was removed \textit{in vacuo} to obtain leucine tert-butyl ester 47 as a colorless oily residue. This residue was dissolved in dry THF (40 mL) and treated with CDI (3.62 g, 22.3 mmol). The resulting solution was heated to reflux for 2 h. The reaction mixture was further treated with DIPEA (5.73 mL, 33.5 mmol) and 4-(aminomethyl)benzonitrile hydrochloride (5.65 g, 33.5 mmol) and heated to reflux for 18 h. The solvent was evaporated, and the residue was suspended in H\textsubscript{2}O. The aqueous suspension was adjusted to pH ~2 with 10% KHSO\textsubscript{4} and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H\textsubscript{2}O (30 mL), 10% KHSO\textsubscript{4} (30 mL), H\textsubscript{2}O (30 mL), and brine (30 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and removed under reduced pressure. The crude product was recrystallized from ethyl acetate to obtain 52 as a white solid (5.72 g, 74%). mp 157 °C; \textsuperscript{1}H NMR (500 MHz, DMSO-\textsubscript{d}\textsubscript{6}) \( \delta \) 0.85 (d, \( J = 6.6 \) Hz, 3H, \( \text{CH}_3\text{CHCH}_3 \)), 0.89 (d, \( J = 6.6 \) Hz, 3H, \( \text{CH}_3\text{CHCH}_3 \)), 1.38 (s, 9H, \( \text{C(CH}_3\text{)}_3 \)), 1.41–1.44 (m, 2H, \( \text{CH}_2\text{CH}_2 \)), 1.59–1.67 (m, 1H, \( \text{CH}_3\text{CHCH}_3 \)), 4.03–4.07 (m, 1H, \( \text{NHCHO} \)), 4.24–4.32 (m, 2H, \( \text{CH}_2\text{NH} \)), 6.28 (d, \( J = 8.5 \) Hz, 1H, \( \text{NHCHO} \)), 6.54 (t, \( J = 6.2 \) Hz, 1H, \( \text{CH}_2\text{NH} \)), 7.41 (d, \( J = 8.5 \) Hz, 2H, H-2', H-6'), 7.76 (d, \( J = 8.5 \) Hz, 2H, H-3', H-5'); \textsuperscript{13}C NMR (125 MHz, DMSO-\textsubscript{d}\textsubscript{6}) \( \delta \) 21.82, 22.80, 24.50 (\( \text{CH}_3\text{CHCH}_3 \), \( \text{CH}_3\text{CHCH}_3 \), \( \text{CH}_3\text{CHCH}_3 \)), 27.78 (\( \text{C(CH}_3\text{)}_3 \)), 41.13, 42.72 (\( \text{CH}_2\text{CH}_2 \), \( \text{CH}_2\text{NH} \)), 51.92 (\( \text{NHCHO} \)), 80.31 (\( \text{C(CH}_3\text{)}_3 \)), 109.43 (C-4'), 119.04 (CN), 127.83 (C-2', C-6'), 132.24 (C-3', C-5'), 147.08 (C-1'), 157.79 (NHCONH), 173.00 (NHCHO); Anal. C\textsubscript{19}H\textsubscript{27}N\textsubscript{3}O\textsubscript{3} (345.44 g/mol) calcd C 66.06, H 7.88, N 12.16; found C 66.46, H 7.90, N 12.30.
3.3.18. PREPARATION OF 53

\[ \text{N-(4-Cyanophenylcarbamoyl)leucine tert-butyl ester.} \]

The hydrochloride salt of 47 (5.00 g, 22.3 mmol) was dissolved in dry THF (50 mL) and treated with DIPEA (5.70 mL, 33.3 mmol). The resulting reaction mixture was treated with 4-cyanophenyl isocyanate (3.55 g, 24.6 mmol) and stirred for 4 h at room temperature. After evaporation of the solvent, the residue was suspended in H₂O. The aqueous suspension was adjusted with 10% KHSO₄ to pH ~2 and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H₂O (20 mL), 10% KHSO₄ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and evaporated under reduced pressure. The crude product was recrystallized from ethyl acetate/petroleum ether to obtain 53 as a white solid (6.70 g, 91%).

mp 113 °C; \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 0.88 (d, \(^3J = 6.6\) Hz, 3H, CH₃CHCH₃), 0.91 (d, \(^3J = 6.7\) Hz, 3H, CH₃CHCH₃), 1.40 (s, 9H, C(CH₃)₃), 1.46–1.54 (m, 2H, CHCH₂), 1.62–1.70 (m, 1H, CH₂CHCH₃), 4.10–4.14 (m, 1H, NHCH₂CO), 6.61 (d, \(^3J = 8.2\) Hz, 1H, NHCH₂CO), 7.54 (d, \(^3J = 8.9\) Hz, 2H, H-3’, H-5’), 7.65 (d, \(^3J = 8.8\) Hz, 2H, H-2’, H-6’), 9.07 (s, 1H, C-1’NH); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 21.82, 22.77, 24.56 (CH₃CHCH₃, CH₃CHCH₃, CH₃CHCH₃), 27.78 (C(CH₃)₃), 40.91 (CHCH₂), 51.64 (NHCH₂CO), 80.85 (C(CH₃)₃), 102.88 (C-4’), 117.68 (C-2’, C-6’), 119.48 (CN), 133.36 (C-3’, C-5’), 144.69 (C-1’), 154.40 (NHCONH), 172.36 (NHCH₂CO); Anal. C₁₈H₂₅N₃O₃ (331.41 g/mol) calcd C 65.23, H 7.60, N 12.68; found C 65.21, H 7.20, N 12.25.
3.3.19. PREPARATION OF 54

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\begin{align*}
\text{N-[(4-Acetoxyamidino)benzylcarbamoyl]leucine tert-butyl ester.} & \quad \text{Compound 52 (2.00 g, 5.79 mmol) was dissolved in EtOH (50 mL) and treated with DIPEA (1.97 mL, 11.5 mmol) and hydroxylamine hydrochloride (0.81 g, 11.7 mmol). The resulting solution was heated to reflux overnight. EtOH was evaporated, the oily residue was dissolved in dry MeCN (50 mL) and treated with acetic anhydride (1.64 mL, 17.5 mmol). The resulting solution was stirred at room temperature for 5 h. The solvent was removed in vacuo, and the oily residue was suspended in H}_2\text{O. The aqueous suspension was adjusted to pH ~2 with 10\% KHSO}_4 \text{ and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with 5\% KHSO}_4 (2 × 20 mL), H}_2\text{O (20 mL), sat. NaHCO}_3 (2 × 20 mL), H}_2\text{O (20 mL), and brine (20 mL). The solvent was dried (Na}_2\text{SO}_4) \text{ and removed under reduced pressure. The solid product was recrystallized from ethyl acetate/}n\text{-hexane to obtain 54 as a white solid (1.86 g, 76\% from 52). mp 148 °C; }^1\text{H NMR (500 MHz, DMSO-}d_6) \delta 0.86 (d, ^3J = 6.6 Hz, 3H, CH}_3\text{CHCH}_3), 0.89 (d, ^3J = 6.6 Hz, 3H, CH}_3\text{CHCH}_3), 1.39 (s, 9H, C(CH}_3)_3), 1.42–1.44 (m, 2H, CHCH}_2), 1.60–1.68 (m, 1H, CH}_3\text{CHCH}_3), 2.12 (s, 3H, CH}_3\text{CO), 4.04–4.09 (m, 1H, NHCHCO), 4.20–4.28 (m, 2H, CH}_2\text{NH), 6.21 (d, ^3J = 8.2 Hz, 1H, NHCHCO), 6.46 (t, }^3J = 6.2 Hz, 1H, CH}_2\text{NH), 6.72 (s, 2H, NH}_2), 7.28, 7.64 (d, ^3J = 8.5 Hz, 2H, d, ^3J = 8.2 Hz, 2H, H-2’, H-6’, H-3’, H-5’); }^{13}\text{C NMR (125 MHz, DMSO-}d_6) \delta 20.00 (\text{CH}_3\text{CO), 21.87, 22.85, 24.54 (\text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3), 27.83 (\text{C(CH}_3)_3), 41.23, 42.68 (\text{CHCH}_2, \text{CH}_2\text{NH), 51.91 (NHCHCO), 80.34 (C(CH}_3)_3), 126.70, 126.88 (C-3’, C-5’, C-2’, C-6’), 130.08 (C-4’), 143.49 (C-1’), 156.41 (C=N), 157.83 (NHCONH), 168.64 (CH}_3\text{CO), 173.10 (NHCHCO); Anal. C}_{21}H_{32}N_4O_5 (420.50 g/mol) calcd C 59.98, H 7.67, N 13.32; found C 59.64, H 7.48, N 13.05.} 
\end{align*}
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3. EXPERIMENTAL SECTION

3.3.20. PREPARATION OF 55

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\text{N-\{4-[(2-Thiophenecarbonyl)oxyamidino]benzylcarbamoyl\}leucine tert-butyl ester.}
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Compound 52 (3.00 g, 8.68 mmol) was dissolved in EtOH (50 mL), and treated with DIPEA (2.98 mL, 17.4 mmol) and hydroxylamine hydrochloride (1.21 g, 17.4 mmol). The resulting solution was heated to reflux overnight. After evaporation of the solvent, the oily residue was suspended in H\textsubscript{2}O. The aqueous suspension was adjusted with 10% KHSO\textsubscript{4} to pH ~2 and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% KHSO\textsubscript{4} (20 mL), H\textsubscript{2}O (20 mL), sat. NaHCO\textsubscript{3} (2 × 20 mL), H\textsubscript{2}O (20 mL), and brine (20 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and removed under reduced pressure. The resulting oily residue (2.48 g, 6.55 mmol) was dissolved in dry MeCN (30 mL) and treated with DIPEA (2.24 mL, 13.1 mmol) and 2-thiophenecarbonyl chloride (1.40 mL, 13.1 mmol). The solution was stirred at room temperature for 5 h. The precipitated product was filtrated and washed with 10% KHSO\textsubscript{4} (3 × 20 mL), H\textsubscript{2}O (20 mL), sat. NaHCO\textsubscript{3} (3 × 20 mL), H\textsubscript{2}O (20 mL), and n-hexane (20 mL). The dry product was recrystallised from ethyl acetate/EtOH (10:3) to obtain 55 as a white solid (2.22 g, 52% from 52). mp 193 °C; \textsuperscript{1}H NMR (500 MHz, DMSO-\textsubscript{d}\textsubscript{6}) \(\delta\) 0.87 (d, \(3J = 6.6\) Hz, 3H, CH\textsubscript{3}CHCH\textsubscript{3}), 0.90 (d, \(3J = 6.7\) Hz, 3H, CH\textsubscript{3}CHCH\textsubscript{3}), 1.40 (s, 9H, C(CH\textsubscript{3})\textsubscript{3}), 1.42–1.45 (m, 2H, CHC\textsubscript{2}H\textsubscript{2}), 1.61–1.69 (m, 1H, CH\textsubscript{2}NCH\textsubscript{2}), 4.05–4.10 (m, 1H, NHC\textsubscript{H}CO), 4.22–4.30 (m, 2H, CH\textsubscript{2}NH), 6.21 (d, \(3J = 8.5\) Hz, 1H, NHCH\textsubscript{2}), 6.21 (d, \(3J = 8.5\) Hz, 1H, NHCH\textsubscript{2}), 6.48 (t, \(3J = 6.2\) Hz, 1H, CH\textsubscript{2}NH), 6.87 (s, 2H, NH\textsubscript{2}), 7.24 (dd, \(3J = 4.9\) Hz, \(3J = 3.6\) Hz, 1H, H-4'''), 7.32, 7.69 (d, \(3J = 8.5\) Hz, 2H, d, \(3J = 8.6\) Hz, 2H, H-2', H-6', H-3', H-5'), 7.96 (dd, \(3J = 4.7\) Hz, \(4J = 1.3\) Hz, 1H, H-5''), 8.12 (dd, \(3J = 3.8\) Hz, \(4J = 1.3\) Hz, 1H, H-3'''), \textsuperscript{13}C NMR (125 MHz, DMSO-\textsubscript{d}\textsubscript{6}) \(\delta\) 21.85, 22.82, 24.51 (CH\textsubscript{3}CHCH\textsubscript{3}, CH\textsubscript{2}CHCH\textsubscript{3}, CH\textsubscript{2}CHCH\textsubscript{3}), 27.81 (C(CH\textsubscript{3})\textsubscript{3}), 41.21, 42.67 (CHCH\textsubscript{2}, CH\textsubscript{2}NH), 51.88 (NHCH\textsubscript{2}), 80.30 (C(CH\textsubscript{3})\textsubscript{3}), 126.87, 126.90, 128.35, 129.94, 132.86, 133.76, 133.88, 143.65 (C-3', C-5', C-4', C-2', C-6', C-4'', C-5'', C-3'', C-2'', C-1'), 156.98 (N=C), 157.79 (NHCONH), 159.79 (C-2''CO), 173.07 (NHCH\textsubscript{2}); Anal. C\textsubscript{24}H\textsubscript{32}N\textsubscript{4}O\textsubscript{5}S (488.60 g/mol) calcd C 59.00, H 6.60, N 11.47; found C 59.04, H 6.51, N 11.44.
3. EXPERIMENTAL SECTION

3.3.21. PREPARATION OF 56

\[ \text{N-[(4-Acetoxyamidino)phenylcarbamoyl]leucine tert-butyl ester.} \]

Compound 53 (6.30 g, 19.0 mmol) was dissolved in EtOH (50 mL), and treated with DIPEA (6.46 mL, 37.7 mmol) and hydroxylamine hydrochloride (2.64 g, 38.0 mmol). The resulting solution was heated to reflux overnight. After evaporation of the solvent, the oily residue was dissolved in dry MeCN (50 mL) and treated with acetic anhydride (5.39 mL, 57.4 mmol). The solution was stirred at room temperature for 5 h. The solvent was removed in vacuo, and the oily residue was suspended in H\textsubscript{2}O. The aqueous suspension was adjusted with 10% KHSO\textsubscript{4} to pH ~2 and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 5% KHSO\textsubscript{4} (2 × 30 mL), H\textsubscript{2}O (30 mL), sat. NaHCO\textsubscript{3} (3 × 30 mL), H\textsubscript{2}O (30 mL), and brine (30 mL). The solvent was dried (\text{Na}_2\text{SO}_4) and removed under reduced pressure. The crude product was recrystallized from ethyl acetate to obtain 56 as a white solid (5.40 g, 70% from 53). mp 95 °C; \textsuperscript{1}H NMR (500 MHz, DMSO-\text{d}_6) \delta 0.89 (d, \textsuperscript{3}J = 6.6 Hz, 3H, \text{CH}_3\text{CHCH}_3), 0.92 (d, \textsuperscript{3}J = 6.6 Hz, 3H, \text{CH}_3\text{CHCH}_3), 1.41 (s, 9H, \text{C(CH}_3)_3), 1.44–1.54 (m, 2H, CHCH\textsubscript{2}), 1.63–1.71 (m, 1H, \text{CH}_3\text{CHCH}_3), 2.11 (s, 3H, \text{CH}_3\text{CO}), 4.11–4.15 (m, 1H, \text{NHCHCO}), 4.15 (d, \textsuperscript{3}J = 8.2 Hz, 1H, \text{NNHCHCO}), 6.45 (d, \textsuperscript{3}J = 8.2 Hz, 1H, \text{NHCHCO}), 6.62 (s, 2H, \text{NH}_2), 7.42, 7.59 (d, \textsuperscript{3}J = 8.9 Hz, 2H, d, \textsuperscript{3}J = 8.9 Hz, 2H, H-3', H-5', H-2', H-6'), 8.74 (s, 1H, C-1'NH); \textsuperscript{13}C NMR (125 MHz, DMSO-\text{d}_6) \delta 20.00 (\text{CH}_3\text{CO}), 21.85, 22.77, 24.55 (\text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3), 27.78 (\text{C(CH}_3)_3), 41.07 (\text{CHCH}_2), 51.56 (\text{NNHCHCO}), 80.73 (\text{C(CH}_3)_3), 116.98 (C-2', C-6'), 124.26 (C-4'), 127.42 (C-3', C-5'), 142.28 (C-1'), 154.66, 156.24 (N=C, \text{NHCONH}), 168.65, 172.56 (\text{CH}_3\text{CO}, \text{NHCHCO}); Anal. C\textsubscript{20}H\textsubscript{30}N\textsubscript{4}O\textsubscript{5} (406.48 g/mol) calcd C 59.10, H 7.44, N 13.78; found C 58.96, H 7.41, N 13.41.
3.3.22. PREPARATION OF 57

\[
\text{N-}{4-\text{[(2-Thiophenecarbonyl)oxyamidino]phenylcarbamoyl}leucine tert-butyl ester.}
\]

Compound 53 (6.10 g, 18.4 mmol) was dissolved in EtOH (50 mL) and treated with DIPEA (6.30 mL, 36.8 mmol) and hydroxylamine hydrochloride (2.56 g, 36.8 mmol). The resulting solution was heated to reflux overnight. After evaporation of the solvent, the oily residue was suspended in H₂O. The aqueous suspension was adjusted to pH ~2 with 10% KHSO₄ and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL), H₂O (30 mL), sat. NaHCO₃ (2 × 30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and removed under reduced pressure. The resulting oily residue (3.96 g, 10.9 mmol) was dissolved in dry MeCN (50 mL), and treated with DIPEA (3.70 mL, 21.6 mmol) and 2-thiophenecarbonyl chloride (1.75 mL, 16.4 mmol). The solution was stirred at room temperature for 5 h. The precipitated product was filtrated and washed with 10% KHSO₄ (3 × 20 mL), H₂O (20 mL), sat. NaHCO₃ (3 × 20 mL), H₂O (20 mL) and n-hexane (20 mL). The dry product was recrystallised from ethyl acetate/EtOH (10:3) to obtain 57 as a white solid (3.58 g, 41% from 53). mp 195–196°C; \(^1\)H NMR (500 Hz, DMSO-\(d_6\)) \(\delta\) 0.90 (d, \(^3J = 6.6\) Hz, 3H, C\(\text{H}_3\)CHCH\(\text{H}_3\)), 0.92 (d, \(^3J = 6.7\) Hz, 3H, CH\(\text{H}_3\)CHC\(\text{H}_3\)), 1.41 (s, 9H, C(CH\(\text{H}_3\))\(\text{S}\)), 1.48–1.52 (m, 2H, CHC\(\text{H}_2\)), 1.64–1.72 (m, 1H, CH\(\text{H}_3\)CHCH\(\text{H}_3\)), 4.12–4.16 (m, 1H, NHC\(\text{H}_2\)CO), 6.47 (d, \(^3J = 4.9\) Hz, 1H, NHCHCO), 6.77 (s, 2H, NH\(\text{H}_2\)), 7.23 (dd, \(^3J = 4.9\) Hz, \(^3J = 3.7\) Hz, 1H, H-4‘’), 7.45, 7.64 (d, \(^3J = 8.8\) Hz, 2H, d, \(^3J = 8.8\) Hz, 2H, H-3’, H-5’, H-2’, H-6’), 7.95 (dd, \(^3J = 5.1\) Hz, \(^4J = 1.3\) Hz, 1H, H-5’’), 8.11 (dd, \(^3J = 3.6\) Hz, \(^4J = 1.4\) Hz, 1H, H-3’’), 8.78 (s, 1H, C-1’NH); \(^1^3\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 21.87, 22.78, 24.57 (CH\(\text{H}_3\)CHCH\(\text{H}_3\), CH\(\text{H}_3\)CHCH\(\text{H}_3\), CH\(\text{H}_3\)CHCH\(\text{H}_3\)), 27.80 (C(CH\(\text{H}_3\))\(\text{S}\)), 41.09 (CHCH\(\text{H}_2\)), 51.58 (NHCHCO), 80.75 (C(CH\(\text{H}_3\))\(\text{S}\)), 117.03 (C-2’, C-6’), 124.12 (C-4’), 127.64 (C-3’, C-5’), 128.34, 132.97, 133.67, 133.80 (C-4’’, C-5’’, C-3’’, C-2’’), 142.45 (C-1’), 154.68 (N=C), 156.86 (NHCONH), 159.83 (C-2’’CO), 172.58 (NHCHCO); Anal. C\(_{23}\)H\(_{30}\)N\(_4\)O\(_5\)S (474.57 g/mol) calcd C 58.21, H 6.37, N 11.81; found C 58.12, H 6.28, N 11.40.
3.3.23. PREPARATION OF 58

N-[4-(5-Methyl-1,2,4-oxadiazol-3-yl)benzylcarbamoyl]leucine. Compound 54 (1.80 g, 4.28 mmol) was dissolved in concentrated acetic acid (40 mL) and stirred at 80 °C for 5 h. After evaporation of the acetic acid, the oily product was dissolved in CH$_2$Cl$_2$ (40 mL) and treated with TFA (20 mL). The resulting solution was stirred for 5 h at room temperature. The solvent was evaporated, and the oily residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (2 × 30 mL) and brine (30 mL). The solvent was dried (Na$_2$SO$_4$) and removed under reduced pressure. The solid product was recrystallized from ethyl acetate/n-hexane to obtain 58 as a white solid (0.43 g, 29% from 54). mp 171 °C; $^1$H NMR (500 MHz, DMSO-d$_6$) δ 0.86 (d, $^3$J = 6.6 Hz, 3H, CH$_3$CHCH$_3$), 0.89 (d, $^3$J = 6.7 Hz, 3H, CH$_3$CHCH$_3$), 1.41–1.51 (m, 2H, CHCH$_2$), 1.61–1.69 (m, 1H, CH$_3$CHCH$_3$), 2.64 (s, 3H, CH$_3$C-5’’), 4.11–4.15 (m, 1H, NHCHCO), 4.23–4.32 (m, 2H, CH$_2$NH), 6.24 (d, $^3$J = 8.5 Hz, 1H, NHCHCO), 6.51 (t, $^3$J = 6.1 Hz, 1H, CH$_2$NH), 7.40 (d, $^3$J = 8.5 Hz, 2H, H-2’, H-6’), 7.92 (d, $^3$J = 8.5 Hz, 2H, H-3’, H-5’), 12.40 (s, 1H, COOH); $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ 12.13 (CH$_3$C-5’’), 21.75, 22.96, 24.50 (CH$_3$CHCH$_3$, CH$_3$CHCH$_3$, CH$_3$CHCH$_3$), 41.18, 42.76 (CHCH$_2$, CH$_2$NH), 51.18 (NHCHCO), 124.76 (C-4’), 127.00, 127.74 (C-3’, C-5’, C-2’, C-6’), 144.67 (C-1’), 157.94 (NHCONH), 167.64, 175.30, 177.46 (C-3’’, NHCHCO, C-5’’); Anal. C$_{17}$H$_{22}$N$_4$O$_4$ (346.38 g/mol) calcd C 58.95, H 6.40, N 16.17; found C 59.05, H 6.26, N 16.01.
3.3.24. PREPARATION OF 60

\[ \text{N-[4-(5-Methyl-1,2,4-oxadiazol-3-yl)phenylcarbamoyl]leucine.} \]

Compound 56 (5.20 g, 12.8 mmol) was dissolved in concentrated acetic acid (40 mL) and stirred at 80 °C for 5 h. After evaporation of the acetic acid, the oily product was dissolved in CH\textsubscript{2}Cl\textsubscript{2} (40 mL) and treated with TFA (20 mL). The resulted solution was stirred for 5 h at room temperature. The solvent was evaporated, and the oily residue was suspended in H\textsubscript{2}O. The resulting aqueous suspension was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H\textsubscript{2}O (2 × 30 mL) and brine (30 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and removed under reduced pressure. The solid product was recrystallized from ethyl acetate/n-hexane to obtain 60 as a white solid (2.80 g, 66% from 56). mp 156–158 °C; \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}\textsubscript{6}) \( \delta \) 0.89–0.92 (m, 6H, CH\textsubscript{(CH\textsubscript{3})\textsubscript{2}}), 1.48–1.58 (m, 2H, CHCH\textsubscript{2}), 1.65–1.73 (m, 1H, CH\textsubscript{(CH\textsubscript{3})\textsubscript{2}}), 2.61 (CH\textsubscript{3}C-5”), 4.17–4.23 (m, 1H, NH\textsubscript{CO}), 6.52 (d, \( ^{3}J = 8.2 \) Hz, 1H, NH\textsubscript{CO}), 7.54 (d, \( ^{3}J = 8.8 \) Hz, 2H, H-2’, H-6’), 7.85 (d, \( ^{3}J = 8.8 \) Hz, 2H, H-3’, H-5’), 8.89 (s, 1H, C-1’NH), 12.62 (bs, 1H, COOH); \textsuperscript{13}C NMR (125 MHz, DMSO-\textit{d}\textsubscript{6}) \( \delta \) 12.09 (CH\textsubscript{3}C-5”), 21.76, 22.94, 24.55 (CH\textsubscript{3}CHCH\textsubscript{3}, CH\textsubscript{3}CHCH\textsubscript{3}, CH\textsubscript{3}CHCH\textsubscript{3}), 41.05 (CHCH\textsubscript{2}), 50.90 (NHCH\textsubscript{CO}), 117.70 (C-2’, C-6’), 119.00 (C-4’), 127.92 (C-3’, C-5”), 143.23 (C-1’), 154.70 (NHCONH), 167.53, 174.80, 177.05, (C-3”, NHCH\textsubscript{CO}, C-5”); Anal. C\textsubscript{16}H\textsubscript{20}N\textsubscript{4}O\textsubscript{4} (332.35 g/mol) calcd C 57.82, H 6.07, N 16.86; found C 57.75, H 6.38, N 15.75.
3.3.25. PREPARATION OF 61

\[
\begin{align*}
\text{N-[4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]phenylcarbamoyl]leucine.} & \quad \text{Compound 57 (3.00 g, 6.32 mmol) was dissolved in concentrated acetic acid (40 mL) and stirred at 80 °C for 5 h.} \\
& \quad \text{After evaporation of the acetic acid, the oily product was dissolved in CH}_2\text{Cl}_2 (40 mL) and treated with TFA (20 mL). The resulting solution was stirred for 3 h at room temperature.} \\
& \quad \text{After evaporation of the solvent, the oily residue was suspended in H}_2\text{O. The aqueous suspension was extracted with ethyl acetate (3 \times 30 mL). The combined organic layers were washed with H}_2\text{O (2 \times 30 mL) and brine (30 mL). The solvent was dried (Na}_2\text{SO}_4) and removed under reduced pressure. The solid product was recrystallized from ethyl acetate/}n\text{-hexane to obtain 61 as a white solid (1.29 g, 51% from 57).} \\
& \quad \text{mp 146–148 °C; } ^1\text{H NMR (500 MHz, DMSO-}d_6\text{) } \delta \text{ 0.90–0.93 (m, 6H, CH(CH}_3)_2\text{), 1.49–1.59 (m, 2H, CHCH}_2\text{), 1.65–1.74 (m, 1H, CH(CH}_3)_2\text{), 4.19–4.23 (m, 1H, NHCHCO), 6.53 (d, }^3\text{J = 8.2 Hz, 1H, NHCHCO),} \\
& \quad 7.35 (dd, }^3\text{J = 5.1 Hz, }^3\text{J = 3.8 Hz, 1H, H-4'''), 7.58 (d, }^3\text{J = 8.8 Hz, 2H, H-2', H-6'), 7.92 (d,} \\
& \quad ^3\text{J = 8.5 Hz, 2H, H-3', H-5'), 8.05, 8.09 (dd, }^3\text{J = 3.8 Hz,}^4\text{J = 1.3 Hz, 1H, dd,}^3\text{J = 5.1 Hz,} \\
& \quad ^4\text{J = 1.0 Hz, 1H, H-5'''', H-3''''), 8.94 (C-1'NH), 12.63 (s, 1H, COOH); } ^{13}\text{C NMR (125 MHz, DMSO-}d_6\text{) } \delta \text{ 21.76, 22.94, 24.56 (CH}_3\text{CHCH}_3\text{, CH}_3\text{CHCH}_3\text{, CH}_3\text{CHCH}_3\text{), 41.02 (CHCH}_2\text{),} \\
& \quad 50.90 (NHCHCO), 117.72 (C-2', C-6'), 118.49 (C-5'''), 124.82 (C-4'), 128.17 (C-3', C-5'), \\
& \quad 129.35, 132.77, 134.01, (C-3'''', C-4'''', C-2''''), 143.52 (C-1'), 154.67 (NHCONH), 168.06, \\
& \quad 170.89, 174.78 (C-3''', NHCHCO, C-5'''');} \\
& \text{Anal. C}_{19}\text{H}_{20}\text{N}_4\text{O}_4\text{S (400.45 g/mol) calcd C 56.99, H 5.03, N 13.99; found C 56.24, H 5.15, N 13.45.}
\end{align*}
\]
3.3.26. PREPARATION OF 62

\[
N\text{-}[4-(5-Methyl-1,2,4-oxadiazol-3-yl)benzylcarbamoyl]leucyl-methylazaalanine-nitrile.}
\]

Compound 58 (0.25 g, 0.72 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. To the stirred solution, \(N\text{-}methyl\text{morpholine (0.09 mL, 0.82 mmol) and isobutyl chloroformate (0.11 mL, 0.84 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (0.48 g, 3.61 mmol) was dissolved in }H_2O (1 mL), and 5 N NaOH (2.00 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of \(N\text{-}methyl\text{morpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with }H_2O (20 mL), sat. NaHCO_3 (20 mL), }H_2O (20 mL), and brine (20 mL). The solvent was dried (Na_2SO_4) and evaporated. The crude product was purified by column chromatography on silica gel using MeOH/CH_2Cl_2 (1:40) to obtain \(N\text{-}[4-(5-methyl-1,2,4-oxadiazol-3-yl)benzylcarbamoyl]leucine 1,2-dimethylhydrazide as a colorless oil. Sodium acetate (0.14 g, 1.71 mmol) and cyanogen bromide (0.07 g, 0.66 mmol) were added to a solution of \(N\text{-}[4-(5-methyl-1,2,4-oxadiazol-3-yl)benzylcarbamoyl]leucine 1,2-dimethylhydrazide (0.26 g, 0.67 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 24 h, and the solvent was removed under reduced pressure. The residue was suspended in }H_2O (10 mL), a pH of ~2 was adjusted (10% KHSO_4), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with }H_2O (20 mL), sat. NaHCO_3 (2 × 20 mL), }H_2O (20 mL), and brine (20 mL). The solvent was dried (Na_2SO_4) and removed \textit{in vacuo}. The oily residue was purified by column chromatography on silica gel using MeOH/CH_2Cl_2 (1:10) as eluent to obtain 62 as a white solid (0.16 g, 54% from 58). mp 68 °C; \([\alpha]^{20}_D = +11.8 \text{ (c = 0.68, }CHCl_3);^1H \text{ NMR (500 MHz, }CDCl_3) \delta 0.95 \text{ (d, }^3J = 6.7 \text{ Hz, 3H, }CH_2CHCH_3), 0.99 \text{ (d, }^3J = 6.3 \text{ Hz, 3H, }CH_3CHCH_3), 1.45\text{–}1.48 \text{ (m, 2H, CHCH}_2), 1.73\text{–}1.79 \text{ (m, 1H, }CH_3CHCH_3), 2.62 \text{ (s, 3H, }CH_3C-5'), 3.00, 3.27 \text{ (2 × s, 2 × 3H, N(CH}_3)CN, CONCH}_3), 4.29 \text{ (dd, }^2J = 15.1 \text{ Hz, }^3J = 5.4 \text{ Hz, 1H, CHHNH), 4.44 \text{ (dd, }^2J = 15.5 \text{ Hz, }^3J = 6.0 \text{ Hz, 1H, C'HNNH}, 4.94\text{–}4.99 \text{ (m, 1H, NHCHCO), 5.58 \text{ (t, }^3J = 5.5 \text{ Hz, 1H, CH}_2NH), 5.74 \text{ (d, }^3J = 8.8 \text{ Hz, 1H, NHCHCO), 7.33 \text{ (d, }^3J = 8.2 \text{ Hz, 2H, H}-2', H'-6'), 7.96 \text{ (d, }^3J = 8.2 \text{ Hz, 2H, H}-3', H'-5');}^{13}C \text{ NMR (125 MHz, }CDCl_3) \delta 12.36 \text{ (CH}_3C-5'), 21.36,
23.29, 24.86 (CH₃CHCH₃, CH₃CHCH₃, CH₃CHCH₃), 30.49 (N(CH₃)CN, 40.86 (CONCH₃),
41.39, 44.00 (CHCH₂, CH₂NH), 48.72 (NHCHCO), 114.47 (CN), 125.73 (C-4'), 127.55,
127.78 (C-3', C-5', C-2', C-6'), 142.37 (C-1'), 158.14 (NHCONH), 168.07, 176.55, 177.05
(C-3'', NHCHCO, C-5''); FTIR (KBr, cm⁻¹) 2223 (C≡N); Anal. C₂₀H₂₇N₇O₃ (413.47 g/mol)
calcd C 58.10, H 6.58, N 23.71; found C 57.62, H 6.47, N 22.97; LC-MS(ESI) (90% H₂O to
100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.3–319.9 nm) tᵣ = 21.02, 99%
purity, m/z = 414.1 ([M + H]⁺).
3. EXPERIMENTAL SECTION

3.3.27. PREPARATION OF 63

\[
\text{N-}[4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzylcarbamoyl]leucyl-methylazaalanine-nitrile.}\]

Compound 55 (2.00 g, 4.09 mmol) was dissolved in concentrated acetic acid (40 mL) and stirred at 80 °C for 5 h. After evaporation of the acetic acid, the oily product was dissolved in CH$_2$Cl$_2$ (40 mL) and treated with TFA (20 mL). The resulting solution was stirred for 3 h at room temperature. The solvent was evaporated, and the oily residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (2 × 30 mL) and brine (30 mL). The solvent was dried (Na$_2$SO$_4$) and removed under reduced pressure. The solid product was recrystallized from ethyl acetate/n-hexane to obtain 59 as a white solid (0.59 g, 35% from 55).

Compound 59 (0.23 g, 0.55 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.07 mL, 0.64 mmol) and isobutyl chloroformate (0.08 mL, 0.61 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (0.37 g, 2.78 mmol) was dissolved in H$_2$O (1 mL), and 5 N NaOH (2.00 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (20 mL), sat. NaHCO$_3$ (20 mL), H$_2$O (20 mL), and brine (20 mL). The solvent was dried (Na$_2$SO$_4$) and evaporated under reduced pressure. The crude product was purified on silica gel using ethyl acetate as eluent to obtain N-\{4-[5-(2-thienyl)-1,2,4-oxadiazol-3-yl]benzylcarbamoyl\}leucine 1,2-dimethylhydrazide as a colorless oil. Sodium acetate (0.09 g, 1.10 mmol) and cyanogen bromide (0.04 g, 0.38 mmol) were added to a suspension of N-\{4-[5-(2-thienyl)-1,2,4-oxadiazol-3-yl]benzylcarbamoyl\}leucine 1,2-dimethylhydrazide (0.17 g, 0.37 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 48 h, and the solvent was removed under reduced pressure. The residue was suspended in H$_2$O (10 mL), a pH of ~2 was adjusted (10% KHSO$_4$), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (20 mL), sat. NaHCO$_3$ (2 × 20 mL), H$_2$O (20 mL), and brine (20 mL). The solvent was dried (Na$_2$SO$_4$) and removed in vacuo. The oily residue was purified by column chromatography on silica gel.
using ethyl acetate/petroleum ether (1:1) as eluent to obtain 63 as a white solid (0.12 g, 45% from 59). mp 96–98 °C. \([\alpha]^{20}_D = +12.8\) (c = 0.86, CHCl₃); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) mixture of rotamers (only the data of the major rotational isomer are noted) δ 0.91–0.94 (m, 6H, CH(CH₃)₂), 1.29–1.47 (m, 2H, CHCH₂), 1.70 (bs, 1H, CH(CH₃)₂), 3.09, 3.22 (2 × s, 2 × 3H, N(CH₃)CN, CONCH₃), 4.30 (d, \(^3\)J = 5.7 Hz, 2H, CH₂NH), 4.74–4.77 (m, 1H, NHCHCO), 6.41 (d, \(^3\)J = 8.2 Hz 1H, NHCHCO), 6.55 (t, \(^3\)J = 5.5 Hz, 1H, CH₂NH), 7.36 (dd, \(^3\)J = 5.1 Hz, \(^3\)J = 3.8 Hz, 1H, H-4’’’), 7.43 (d, \(^3\)J = 8.2 Hz, 2H, H-2’, H-6’), 7.99 (d, \(^3\)J = 8.6 Hz, 2H, H-3’, H-5’), 8.07, 8.10 (dd, \(^3\)J = 3.8 Hz, \(^4\)J = 1.3 Hz, 1H, dd, \(^3\)J = 5.1 Hz, \(^4\)J = 1.3 Hz, 1H, H-5’’’; H-3’’’); \(^13\)C NMR (125 MHz, DMSO-\(d_6\)) δ 21.45, 23.31, 24.52 (CH₃CH₃, CH₃CHCH₃, CH₃CHCH₃), 30.50 (N(CH₃)CN), CONCH₃ obscured by the DMSO signal, 40.96 (CH₂CH₂), 42.79 (CH₂NH), 48.08 (NHCHCO), 114.30 (CN), 124.36, 124.67, 127.24, 127.79, 129.39, 132.93, 134.18, 144.85 (C-5’’’, C-3’’’, C-3’, C-5’, C-2’, C-6’, C-4’’, C-4’, C-2’’, C-1’), 158.07 (NHCONH), 168.16, 171.19, 175.29 (C-3’’, NHCHCO, C-5’’’); FTIR (KBr, cm⁻¹) 2222 (C≡N); Anal. C\(_{23}\)H\(_{27}\)N\(_7\)O\(_3\)S (481.57 g/mol) calcd C 57.36, H 5.65, N 20.36; found C 56.63, H 5.80, N 19.43. LC-MS(ESI) (90% H\(_2\)O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 199.6–300.0 nm) \(t_r = 23.00\), 95% purity, \(m/z = 482.1\) ([M + H]⁺).
3. EXPERIMENTAL SECTION

3.3.28. PREPARATION OF 64

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\text{N-[4-(5-Methyl-1,2,4-oxadiazol-3-yl)phenylcarbamoyl]leucyl-methylazaalanine-nitrile.}
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Compound 60 (1.50 g, 4.51 mmol) was dissolved in dry THF (40 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.54 mL, 4.91 mmol) and isobutyl chloroformate (0.65 mL, 4.99 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (3.00 g, 22.6 mmol) was suspended in H\textsubscript{2}O (3 mL), and 10 N NaOH (5.00 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H\textsubscript{2}O (30 mL), sat. NaHCO\textsubscript{3} (30 mL), H\textsubscript{2}O (30 mL), and brine (30 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and evaporated. The crude product was purified by column chromatography on silica gel using ethyl acetate to obtain N-[4-(5-methyl-1,2,4-oxadiazol-3-yl)phenylcarbamoyl]leucine 1,2-dimethylhydrazide as a colorless oil. Sodium acetate (0.48 g, 5.85 mmol) and cyanogen bromide (0.47 g, 4.44 mmol) were added to a solution of N-[4-(5-methyl-1,2,4-oxadiazol-3-yl)phenylcarbamoyl]leucine 1,2-dimethylhydrazide (1.10 g, 2.94 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 48 h, and the solvent was removed under reduced pressure. The residue was suspended in H\textsubscript{2}O (10 mL), a pH of ~2 was adjusted (10% KHSO\textsubscript{4}), and it was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H\textsubscript{2}O (20 mL), sat. NaHCO\textsubscript{3} (2 × 30 mL), H\textsubscript{2}O (30 mL), and brine (30 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using MeOH/CH\textsubscript{2}Cl\textsubscript{2} (40:1) as eluent. Additionally, the product was recrystallized from ethyl acetate/petroleum ether to obtain 64 as a white solid (0.66 g, 37% from 60). mp 185 °C; [\alpha]^{20}_D = +65.6 (c = 0.32, CHCl\textsubscript{3}); \textsuperscript{1}H NMR (500 MHz, DMSO-d\textsubscript{6}) mixture of rotamers (only the data of the major rotational isomer are noted) δ 0.93–0.97 (m, 6H, CH(C\textsubscript{6}H\textsubscript{3})), 1.36–1.53 (m, 2H, CHCH\textsubscript{2}), 1.73 (bs, 1H, CH(CH\textsubscript{3})\textsubscript{2}), 2.62 (s, 3H, CH\textsubscript{3}C-5’’), 3.11, 3.27 (2 × s, 2 × 3H, N(CH\textsubscript{3})CN, CONCH\textsubscript{3}), 4.80–4.83 (m, 1H, NHCO), 6.64 (d, \textsuperscript{3}J = 8.2 Hz, 1H, NHCH\textsubscript{2}CO), 7.53 (d, \textsuperscript{3}J = 8.5 Hz, 2H, H-2’, H-6’), 7.85 (d, \textsuperscript{3}J = 8.8 Hz, 2H, H-3’, H-5’), 8.89 (s, 1H, C-1’NH); \textsuperscript{13}C NMR (125 MHz, DMSO-d\textsubscript{6}) δ 12.09 (CH\textsubscript{3}C-5’’), 21.40, 23.29, 24.57 (CH\textsubscript{3}CH\textsubscript{3}, CH\textsubscript{3}CH\textsubscript{2}CH\textsubscript{3}, CH\textsubscript{3}CH\textsubscript{2}CH\textsubscript{3}), 30.56 (N(CH\textsubscript{3})CN), 40.46, 40.94 (CONCH\textsubscript{3},...
CHCH₂, 47.91 (NHCHCO), 114.22 (CN), 117.79 (C-2’, C-6’), 119.15 (C-4’), 127.92 (C-3’, C-5’), 142.98 (C-1’), 154.93 (NHCONH), 167.50, 174.67, 177.07 (C-3”, NHCHCO, C-5”); FTIR (KBr, cm⁻¹) 2219 (C≡N); Anal. C₁₀H₂₃N₇O₃ (399.45 g/mol) calcd C 57.13, H 6.31, N 24.55; found C 57.05, H 6.72, N 24.06; LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 239.8–340.8 nm) tᵣ = 21.83, 98% purity, m/z = 400.3 ([M + H]⁺).
3.3.29. PREPARATION OF 65

*N*-{4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]phenylcarbamoyl}leucyl-methylazalanine-nitrile. Compound 61 (0.65 g, 1.62 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. To the stirred solution, *N*-methylmorpholine (0.20 mL, 1.82 mmol) and isobutyl chloroformate (0.24 mL, 1.84 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (1.07 g, 8.04 mmol) was suspended in H₂O (1 mL), and 5 N NaOH (3.40 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of *N*-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), sat. NaHCO₃ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using ethyl acetate as eluent to obtain *N*-{4-[5-(2-thienyl)-1,2,4-oxadiazol-3-yl]phenylcarbamoyl}leucine 1,2-dimethylhydrazide as a colourless oil. Sodium acetate (0.28 g, 3.41 mmol) and cyanogen bromide (0.16 g, 1.51 mmol) were added to a solution of *N*-{4-[5-(2-thienyl)-1,2,4-oxadiazol-3-yl]phenylcarbamoyl}leucine 1,2-dimethylhydrazide (0.61 g, 1.38 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 48 h, and the solvent was removed under reduced pressure. The residue was suspended in H₂O (10 mL), a pH of ~2 was adjusted (10% KHSO₄) and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), sat. NaHCO₃ (2 × 20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate as eluent to obtain 65 as a white solid (0.51 g, 67% from 61). mp 92 °C; [α]ₓ²⁰₀ = +53.0 (c = 1.05, CHCl₃); ¹H NMR (500 MHz, DMSO-d₆) mixture of rotamers (only the data of the major rotational isomer are noted) δ 0.94 (d, ³J = 6.7 Hz, 3H, CH₃CHCH₃), 0.97 (d, ³J = 6.0 Hz, 3H, CH₃CHCH₃), 1.37–1.55 (m, 2H, CHCH₂), 1.74 (bs, 1H, CH₃CHCH₃), 3.12, 3.27 (2 × s, 2 × 3H, N(CH₃)CN, CONCH₃), 4.81–4.84 (m, 1H, NHCHCO), 6.65 (d, ³J = 7.9 Hz, 1H, NHC™CNCO), 7.35 (dd, ³J = 5.0 Hz, ³J = 3.8 Hz, 1H, H-4'''), 7.57 (d, ³J = 8.8 Hz, 2H, H-2', H-6'), 7.92 (d, ³J = 8.9 Hz, 2H, H-3', H-5'), 8.05, 8.09 (dd, ³J = 3.8 Hz, ⁴J = 1.0 Hz, 1H, dd, ³J = 4.9 Hz,
$^{4}J = 1.1 \text{ Hz, } 1\text{H, H-5''', H-3'''}$, 8.95 (s, 1H, C-1’NH); $^{13}\text{C NMR (125 MHz, DMSO-}d_{6})$
$\delta$ 21.39, 23.29, 24.58 (CH$_{3}$CHCH$_{3}$, CH$_{3}$CHCH$_{3}$, CH$_{3}$CHCH$_{3}$), 30.57 (N(CH$_{3}$)CN), 40.48 (CONCH$_{3}$), 40.94 (CHCH$_{2}$), 47.93 (NHCHCO), 114.21 (CN), 117.79 (C-2’, C-6’), 118.64 (C-5’), 124.79 (C-4’), 128.17 (C-3’, C-5’), 129.34, 132.77, 134.00 (C-3’’, C-4’’, C-2’’’), 143.27 (C-1’), 154.89 (NHCONH), 168.03, 170.90, 174.65 (C-3’’, NHCHCO, C-5’’’); FTIR (KBr, cm$^{-1}$) 2224 (C≡N). Anal. C$_{22}$H$_{25}$N$_{7}$O$_{3}$S (467.54 g/mol) calcd C 56.52, H 5.39, N 20.97; found C 56.41, H 5.77, N 19.94; LC-MS(ESI) (90% H$_2$O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) $t_r = 21.70$, 99% purity, $m/z = 468.5$ ([M + H]$^+$).
3. EXPERIMENTAL SECTION

3.3.30. PREPARATION OF 66

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\begin{array}{c}
\text{N-\[4-\{5-(2-Thienyl)-1,2,4-oxadiazol-3-yl\]phenylcarbamoyl\}leucyl-glycine-nitrile.}
\end{array}
\]

Compound 61 (1.00 g, 2.50 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.30 mL, 2.73 mmol) and isobutyl chloroformate (0.36 mL, 2.76 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.31 g, 8.50 mmol) was dissolved in H\(_2\)O (2 mL), and 5 N NaOH (3.00 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 x 20 mL). The combined organic layers were washed with H\(_2\)O (20 mL), sat. NaHCO\(_3\) (20 mL), H\(_2\)O (20 mL), and brine (20 mL). The solvent was dried (Na\(_2\)SO\(_4\)) and evaporated under reduced pressure. The oily crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether as eluent to obtain 66 as a white solid (0.29 g, 26%). mp 237 °C; \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 0.89–0.92 (m, 6H, CH\((CH_3)_2\)), 1.42–1.52 (m, 2H, CHCH\(_2\)), 1.59–1.67 (m, 1H, CH\((CH_3)_2\)), 4.15 (d, \(^3J = 6.3\) Hz, 2H, NHCH\(_2\)CN), 4.24–4.29 (m, 1H, NHCHCO), 6.52 (d, \(^3J = 8.2\) Hz, 1H, NHCHCO), 7.35 (dd, \(^3J = 5.1\) Hz, \(^3J = 3.8\) Hz, 1H, H-4'''''), 7.58 (d, \(^3J = 8.9\) Hz, 2H, H-2', H-6'), 7.92 (d, \(^3J = 8.9\) Hz, 2H, H-3', H-5'), 8.05, 8.09 (dd, \(^3J = 3.6\) Hz, \(^4J = 1.1\) Hz, 1H, dd, \(^3J = 5.1\) Hz, \(^4J = 1.3\) Hz, 1H, H-5'''', H-3'''''), 8.83 (t, \(^3J = 5.7\) Hz, 1H, NHCH\(_2\)CN), 8.93 (s, 1H, C-1'NH); \(^13\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 21.91, 23.06, 24.43 (CH\(_3\)CHCH\(_3\), CH\(_3\)CHCH\(_3\), CH\(_3\)CHCH\(_3\)), 27.21 (NHCH\(_2\)CN), 41.79 (CHCH\(_2\)), 51.29 (NHCHCO), 117.65 (CN), 117.70 (C-2', C-6'), 118.51 (C-5'''''), 124.81 (C-4'), 128.19 (C-3', C-5'), 129.36, 132.77, 134.01 (C-3'''', C-4'''', C-2''''), 143.49 (C-1'), 154.48 (NHCONH), 168.05, 170.90, 173.44 (C-3'', NHCHCO, C-5'''); FTIR (KBr, cm\(^{-1}\)) 2253 (C≡N); Anal. C\(_{21}\)H\(_{22}\)N\(_6\)O\(_3\)S (438.50 g/mol) calcd C 57.52, H 5.06, N 19.17; found C 57.50, H 5.15, N 19.02; LC-MS(ESI) (90% H\(_2\)O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) \(t_r = 20.08\), 100% purity, \(m/z = 439.3\) ([M + H]\(^+\)).
3.3.31. PREPARATION OF 67

N-{4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl}-leucyl-methylazaalanine-nitrile.

Sodium acetate (0.19 g, 2.32 mmol) and cyanogen bromide (0.19 g, 1.79 mmol) were added to a solution of 89 (0.50 g, 1.17 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 48 h, and the solvent was removed under reduced pressure. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum (1:1) as eluent to obtain 67 (0.28 g, 53%). mp 92–94 °C; [α]$_{D}^{20} = +47.7$ (c = 1.24, CHCl$_3$); $^1$H NMR (500 MHz, DMSO-$d_6$) mixture rotamers (only the data of the major rotational isomer are noted) δ 0.96 (d, $^3$J = 6.4 Hz, 6H, CH(CH$_3$)$_2$), 1.37–1.44 (m, 1H, CH(CH$_3$)$_2$), 1.72–1.89 (m, 2H, CHCH$_2$), 3.13, 3.32 (2 × s, 2 × 3H, N(CH$_3$)CN, CONCH$_3$), 5.04 (bs, 1H, NHCHCO), 7.37 (dd, $^3$J = 5.1 Hz, $^3$J = 3.8 Hz, 1H, H-4''), 8.08–8.10, 8.12 (m, 3H, dd, $^3$J = 5.1 Hz, $^4$J = 1.3 Hz, 1H, H-3''', H-3', H-5', H-5'''), 8.15 (d, $^3$J = 8.2 Hz, 2H, H-2', H-6'), 8.95 (d, $^3$J = 7.0 Hz, NHCHCO); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 21.14, 23.24, 24.73 (CH$_3$CH$_3$, CH$_3$CHCH$_3$, CH$_3$CHCH$_3$, 30.55 (N(CH$_3$)CN), CONCH$_3$ obscured by the DMSO, 40.89 (CHCH$_2$), 48.74 (NHCHCO), 114.29 (CN), 124.52 (C-5'''), 127.19, 128.63, 129.43, 133.13, 134.40, 136.41 (C-3', C-5', C-2', C-6', C-3''', C-4'''', C-2''', C-4', C-1'), 166.27, 167.77, 171.50, 174.03 (C-3'', C-1'CO, NHCHCO, C-5'''); FTIR (KBr, cm$^{-1}$) 2223 (C≡N); Anal. C$_{22}$H$_{24}$N$_6$O$_5$S (452.53 g/mol) calcd C 58.39, H 5.35, N 18.57; found C 58.41, H 5.84, N 17.55; LC-MS(ESI) (90% H$_2$O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) t$_r$ = 18.99, 95% purity, m/z = 453.3 ([M + H]$^+$).
3.3.32. PREPARATION OF 68

N-[4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl]-N-methylleucyl-methylazaalanine-nitrile. Sodium acetate (0.26 g, 3.17 mmol) and cyanogen bromide (0.25 g, 2.36 mmol) were added to a solution of 90 (0.70 g, 1.59 mmol) in MeOH (30 mL). The mixture was stirred at room temperature for 31 h, and the solvent was removed under reduced pressure. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:2) as eluent to obtain 68 as a white solid (0.46 g, 62%). mp 92–94 °C; $\left[\alpha\right]_{D}^{20} = +3.8$ (c = 1.20, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$) mixture of s-cis and s-trans isomers $\delta$ 0.86$_a$, 1.00$_b$, 1.06$_c$, 1.09$_d$ (bs$_a$, db$_b$, $^3$J = 6.0 Hz, dc$_c$, $^3$J = 6.6 Hz, d$_d$, $^3$J = 6.3 Hz 6H, CH(CH$_3$)$_2$) 1.54–1.60$_a$, 1.67–1.77$_b$ (2 × m, 2H, CHCH$_2$), 1.95–2.01 (bs, 1H, CH(CH$_3$)$_2$), 2.49, 2.81, 3.11, 3.20, 3.23, 3.36 (6 × s, 9H, CH$_3$NCHCO, N(CH$_3$)CN, CONCH$_3$), 5.64–5.67$_a$, 5.78$_b$ (m$_a$, bs$_b$, 1H, CH$_3$NCHCO), 7.21 (dd, $^3$J = 4.8 Hz, $^3$J = 3.8 Hz, 1H, H-4''), 7.52$_a$, 7.65–7.68$_b$, 7.95$_c$, 8.18$_d$ (da$_a$, $^3$J = 8.2 Hz, mb$_b$, 3H, dd$_c$, $^3$J = 3.7 Hz, $^4$J = 0.80 Hz, 1H, dd$_d$, $^3$J = 8.2 Hz, 2H, H-3', H-5', H-5'', H-3''', H-2', H-6'); $^{13}$C NMR (125 MHz, DMSO-d$_6$) mixture of s-cis and s-trans isomers (only the data of the major rotational isomer are noted) $\delta$ 21.19, 23.23, 25.71 (CH$_2$CHCH$_3$, CH$_3$CHCH$_3$, CH$_3$CHCH$_3$), 30.28, 33.97 (N(CH$_3$)CN, CH$_3$NCHCO), 37.42 (CONCH$_3$), 40.73 (CHCH$_2$), 51.52 (CH$_3$NCHCO), 113.80 (CN), 125.63, 127.65, 127.70, 128.20, 128.56, 132.05, 132.19, 138.61 (C-4', C-3', C-5', C-4''', C-5''', C-2', C-6', C-3'''', C-2''', C-1'), 168.15, 171.61. 172.57, 174.34 (C-3''', C-1'CO, CH$_3$NCHCO, C-5''); FTIR (KBr, cm$^{-1}$) 2219 (C≡N); Anal. C$_{23}$H$_{26}$N$_6$O$_3$S (466.56 g/mol) calcd C 59.21, H 5.62, N 18.01; found C 59.27, H 5.61, N 17.19; LC-MS(ESI) (90% H$_2$O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) $t_r = 19.66$, 96% purity, $m/z = 467.6$ ([M + H$^+$]).
3.3.3. PREPARATION OF 69

4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]-benzoic acid. Compound 74 (2.00 g, 5.77 mmol) was suspended in concentrated acetic acid (70 mL). The resulting reaction mixture was stirred at 80 °C overnight. Acetic acid was removed. The residue was dissolved in CH$_2$Cl$_2$ (40 mL) and treated with TFA (5 mL). The reaction mixture was stirred at room temperature for 6 h. The solvent was evaporated under reduced pressure. The crude product was recrystallized from ethyl acetate/petroleum ether to obtain 69 (1.31 g, 83% from 74). mp 232 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) 7.36 (dd, $^3$J = 5.1 Hz, $^3$J = 3.8 Hz, 1H, H-4'''), 8.09, 8.11–8.13 (dd, $^3$J = 3.8 Hz, $^3$J = 1.3 Hz, 1H, m, 3H, H-5''', H-3', H-5', H-3'''), 8.16 (d, $^3$J = 8.9 Hz, 2H, H-2', H-6'), 13.23 (s, 1H, COOH); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 124.50 (C-5'''), 127.51 (C-3', C-5'), 129.46, 129.82 (C-3''', C-4'''), 130.29 (C-2', C-6'), 133.17, 133.63, 134.46 (C-1', C-2'''', C-4'''), 166.75, 167.72, 171.57 (C-3'', COOH, C-5'''). Anal. C$_{13}$H$_8$N$_2$O$_3$S (272.28 g/mol) calcd C 57.35, H 2.96, N 10.29; found C 57.37, H 2.88, N 10.18.
3.3.34. PREPARATION OF 73

4-Cyanobenzoic acid tert-butyl ester. 4-Cyanobenzoic acid 72 (4.50 g, 30.6 mmol) was dissolved in dry CH$_2$Cl$_2$ (100 mL). Oxalyl chloride (4.00 mL, 47.3 mmol) and DMF (1 mL) were added. The resulting reaction mixture was stirred at room temperature for 1 h until the gas evolution was ceased. The solvent was removed. The resulting residue was treated with 60 mL of a pyridine/tert-butanol mixture (1:1) and stirred for 6 h at room temperature. The solvent was evaporated under reduced pressure, and the green residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with 10% KHSO$_4$ (2 × 50 mL), H$_2$O (50 mL), sat. NaHCO$_3$ (2 × 50 mL), H$_2$O (50 mL), and brine (2 × 50 mL). The solvent was dried (Na$_2$SO$_4$) and evaporated. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:4) to obtain 73 as a white solid (4.81 g, 77%). mp 78 °C; $^1$H NMR (500 Hz, DMSO-$d_6$) $\delta$ 1.54 (s, 3H, C(CH$_3$)$_3$), 7.96 (d, $^3$J = 8.9 Hz, 2H, H-3’, H-5’), 8.03 (d, $^3$J = 8.8 Hz, 2H, H-2’, H-6’); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 28.52 (C(CH$_3$)$_3$), 82.81 (C(CH$_3$)$_3$), 115.99 (C-4’), 118.98 (CN), 130.52 (C-2’, C-6’), 133.54 (C-3’, C-5’), 136.06 (C-1’), 164.47 (COO); Anal. C$_{12}$H$_{13}$NO$_2$ (203.24 g/mol) calcd C 70.92, H 6.45, N 6.89; found C 70.72, H 6.43, N 6.91.
3.3.35. PREPARATION OF 74

![Chemical structure](image)

4-[(2-Thiophenecarbonyl)oxyamidino]-benzoic acid tert-butyl ester. Compound 73 (2.00 g, 9.84 mmol) was dissolved in EtOH (70 mL). Triethylamine (2.54 mL, 18.2 mmol) and hydroxylamine hydrochloride (1.27 g, 18.3 mmol) were added. The resulting reaction mixture was heated to reflux for 3 h. EtOH was removed, the oily residue was suspended in H₂O and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with 10% KHSO₄ (50 mL), H₂O (50 mL), brine (50 mL), and dried over Na₂SO₄. After evaporation of the solvent, the white solid residue was dissolved in dry MeCN (40 mL). The resulting reaction mixture was treated under ice-cooling with triethylamine (2.54 mL, 18.2 mmol) and 2-thiophenecarbonyl chloride (1.96 mL, 18.3 mmol), and stirred for 5 h at room temperature. The solvent was evaporated under reduced pressure, and the white residue was suspended in H₂O. The aqueous suspension was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with 10% KHSO₄ (2 × 50 mL), H₂O (50 mL), sat. NaHCO₃ (2 × 50 mL), H₂O (50 mL), and brine (2 × 50 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The crude product was recrystallized from ethyl acetate/petroleum ether to obtain 74 as a white solid (2.60 g, 76% from 73). mp 170 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 1.56 (s, 9H, C(CH₃)₃), 7.05 (s, 2H, NH₂), 7.25 (dd, ³J = 5.1 Hz, ²J = 3.8 Hz, 1H, H-4''), 7.87, 7.96–7.98 (d, ³J = 8.8 Hz, 2H, m, 3H, H-3', H-5', H-2', H-6', H-5''), 8.15 (dd, ³J = 3.8 Hz, ⁴J = 1.3 Hz, 1H, H-3''); ¹³C NMR (125 MHz, DMSO-d₆) δ 27.90 (C(CH₃)₃), 81.30 (C(CH₃)₃), 127.26 (C-3', C-5'), 128.40 (C-4'''), 129.13 (C-2', C-6'), 132.64, 133.19, 133.96, 134.06 135.73 (C-1', C-4', C-5'', C-3'', C-2'''); 156.39, 159.70, 164.57 (C-2''CO, N=C, C-1'CO); Anal. C_{17}H_{18}N_{2}O_{4}S (346.40 g/mol) calcd C 58.94, H 5.24, N 8.09; found C 59.04, H 5.30, N 8.07.
3. EXPERIMENTAL SECTION

3.3.36. PREPARATION OF 77

![Chemical Structure of Leucyl-glycine-nitrile]

**Leucyl-glycine-nitrile.** Compound 75 (5.00 g, 21.6 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (2.61 mL, 23.7 mmol) and isobutyl chloroformate (3.10 mL, 23.8 mmol) were added consecutively. Aminoacetonitrile monosulfate (6.65 g, 43.1 mmol) was suspended in H₂O (3 mL). The resulting suspension was treated with 5 N NaOH (17.0 mL) under ice-cooling and added to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL), H₂O (30 mL), sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and removed *in vacuo* to obtain *N*-(*tert*-butyloxycarbonyl)leucyl-glycine-nitrile as a white solid (5.78 g, 99%). *N-*(*tert*-Butyloxycarbonyl)leucyl-glycine-nitrile (5.78 g, 21.5 mmol) was dissolved in dry THF (20 mL) and treated with methanesulfonic acid (8.40 mL, 0.13 mol). The reaction mixture was stirred at room temperature for 30 min. THF was removed under reduced pressure, and the oily residue was dissolved in H₂O (10 mL). The obtained solution was adjusted at pH ~12 with 2 N NaOH and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL) and dried over Na₂SO₄. The solvent was evaporated, and the oily residue was purified by column chromatography using MeOH/CH₂Cl₂ (1:20) to obtain 77 as an oily product (1.20 g, 33% from *N*-(*tert*-butyloxycarbonyl)leucyl-glycine-nitrile).

**1H NMR** (500 MHz, DMSO-½₆) δ 0.84 (d, J = 6.6 Hz, 3H, CH₃CHCH₃), 0.87 (d, J = 6.6 Hz, 3H, CH₃CHCH₃), 1.20–1.42 (m, 2H, CH₂CH₂), 1.66–1.74 (CH₃CHCH₃), 3.17–3.20 (NH₂C₃H₂), 4.09 (s, 2H, NHCH₂CN); **13C NMR** (125 MHz, DMSO-½₆) δ 21.87, 23.27, 24.17 (CH₃CHCH₃, CH₂CHCH₂, CH₂CHCH₃), 27.09 (NH₂CH₂CN), 44.26 (CH₂CH₂), 53.11 (NH₂CHCO), 117.83 (CN), 176.65 (NH₂CHCO).
3.3.37. PREPARATION OF 79

\[ \text{N-}[4-\{5-\{2-\text{Thienyl}\}-1,2,4-\text{oxadiazol-3-yl\}]\text{benzoyl}\}-\text{leucyl-glycine-nitrile}. \]

Compound 69 (1.32 g, 4.85 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.59 mL, 5.37 mmol) and isobutyl chloroformate (0.70 mL, 5.37 mmol) were added consecutively. Compound 77 (1.10 g, 6.50 mmol) in dry THF (10 mL) was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), sat. NaHCO₃ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:2) as eluent to obtain 79 as a white solid (1.01 g, 49% from 69). mp 195 °C; \([\alpha]^{20}_{D} = -23.9 \text{ (c = 1.34, CHCl₃)}; \] ¹H NMR (500 MHz, DMSO-d₆) \(\delta\) 0.88 (d, \(3J = 6.3\) Hz, 3H, CH₃CHCH₃), 0.92 (d, \(3J = 6.6\) Hz, 3H, CH₃CHCH₃), 1.54–1.60 (m, 1H, CH₃CHCH₃), 1.63–1.77 (m, 2H, CHCH₃), 4.14 (d, \(3J = 5.7\) Hz, 2H, NHCH₂CN), 4.52–4.57 (m, 1H, NHCHCO), 7.37 (dd, \(3J = 5.1\) Hz, \(3J = 3.8\) Hz, 1H, H-4''), 8.09–8.13 (m, 4H, H-3', H-5', H-3'''', H-5'''''), 8.15 (d, \(3J = 8.5\) Hz, 2H, H-2', H-6'), 8.73 (t, \(3J = 5.5\) Hz, 1H, NHCH₂CN), 8.76 (d, \(3J = 8.2\) Hz, 1H, NHCHCO); ¹³C NMR (125 MHz, DMSO-d₆) \(\delta\) 21.41, 23.13, 24.56 (CH₃CHCH₃, CH₃CHCH₃, CH₃CHCH₃), 27.30 (NHCH₂CN), CHCH₂ obscured by the DMSO signal), 51.81 (NHCHCO), 117.71 (CN), 124.53 (C-5'''''), 127.12 (C-3', C-5'), 128.53 (C-3'''''), 128.69 (C-2', C-6'), 129.44, 133.14, 134.41, 136.79 (C-4''''', C-2''''', C-4', C-1'), 165.83, 167.80, 171.51, 172.94 (C-3'''', C-1'CO, NHCHCO, C-5'''); FTIR (KBr, cm⁻¹) 2258 (C≡N); Anal. C₂₁H₂₁N₅O₃S (423.49 g/mol) calcd C 59.56, H 5.00, N 16.54; found C 59.45, H 4.99, N 16.08. LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) \(t_r = 18.92, 98\%\) purity, \(m/z = 424.3\) ([M + H]⁺).
**3.38. PREPARATION OF 80**

![Chemical Structure](image)

*N*-{4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl}-N-methylleucyl-glycine-nitrile. Compound 76 (1.00 g, 4.08 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. To the stirred solution, *N*-methylmorpholine (0.49 mL, 4.46 mmol) and isobutyl chloroformate (0.61 mL, 4.68 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.57 g, 10.2 mmol) was dissolved in H₂O (2 mL) and treated with 10 N NaOH (2 mL). This solution was given to the reaction mixture when the precipitation of *N*-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), 10% KHSO₄ (20 mL), H₂O (20 mL), sat. NaHCO₃ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and evaporated *in vacuo* to obtain *N*-(tert-butyloxycarbonyl)-*N*-methylleucyl-glycine-nitrile as a yellow oil. *N*-(tert-Butoxy carbonyl)-*N*-methylleucyl-glycine-nitrile (1.17 g, 3.93 mmol) was dissolved in dry THF (20 mL) and treated with methanesulfonic acid (0.79 mL, 12.2 mmol). The resulting solution was stirred for 5 h at room temperature. The solvent was removed under reduced pressure. The oily residue was dissolved in H₂O (10 mL). The aqueous solution was adjusted at pH ~12 with 2 N NaOH and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H₂O (20 mL), sat. NaHCO₃ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and evaporated *in vacuo* to obtain 78 as a yellow oil. Compound 69 (0.87 g, 3.20 mmol) was dissolved in dry THF (20 mL). To the stirred solution, DMAP (0.026 g, 0.21 mmol) and EDC (0.83 mL, 4.69 mmol) were added consecutively. The resulting reaction mixture was stirred for 15 min at room temperature. Compound 78 (0.86 g, 4.69 mmol) was added. It was stirred for 18 h at rt. The solvent was removed, and the oily residue was suspended in H₂O. The aqueous suspension was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H₂O (20 mL), 10% KHSO₄ (20 mL), H₂O (20 mL), sat. NaHCO₃ (20 mL), H₂O (20 mL), and brine (20 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 80 as a yellow solid (0.60 g, 43% from 69). mp 68–72 °C;
[α]_D^{20} = -72.1 (c = 1.04, CHCl₃); $^1$H NMR (500 MHz, DMSO-d₆) mixture of s-cis and s-trans isomers δ 0.60ₐ, 0.82ₘ, 0.94ₜ, 0.98ₜ (dₐ, $^3$J = 4.8 Hz, bsₘ, dₜ, $^3$J = 6.3 Hz, dₜ, $^3$J = 6.4 Hz, 6H CH₃CHCH₃, CH₂CHCH₂), 1.21ₐ, 1.39ₘ, 1.58ₜ, 1.70–1.76ₜ (bsₐ, bsₘ, bsₜ, mₜ, 3H, CH₃CHCH₃, CHCH₂), 2.83ₐ, 2.94ₘ (2 × s, 3H, NCH₃), 4.09ₐ, 4.16ₘ (dₐ, $^3$J = 4.8 Hz, dₘ, $^3$J = 5.4 Hz, 2H, NHCH₂CN), 5.12–5.15 (m, 1H, CH₃NCHCO), 7.37 (dd, $^3$J = 5.0 Hz, $^3$J = 3.8 Hz, 1H, H-4‴″), 7.51ₐ, 7.63ₘ (dₐ, $^3$J = 6.9 Hz, dₘ, $^3$J = 7.9 Hz, 2H, H-3′, H-5′), 8.09–8.14 (m, 4H, H-2′, H-6′, H-3‴″, H-5‴″), 8.72ₐ, 8.78ₘ (2 × bs, 1H, NHCH₂CN); $^{13}$C NMR (125 MHz, DMSO-d₆) mixture of s-cis and s-trans isomers δ 21.15, 21.57, 22.99, 23.28, 24.40, 24.71 (CH₃CHCH₃, CH₃CHCH₃, CH₂CHCH₂), 27.42, 29.35 (NHCH₂CN), 33.46, 36.78 (NCH₃), 37.72, 40.90 (CHCH₂), 54.30, 59.96 (CH₃NCHCO), 117.54, 117.67 (CN), 124.54, 126.98, 127.37, 127.46, 127.98, 129.43, 133.10, 134.37, 139.33 (C-5‴″, C-3′, C-5′, C-3‴″, C-2′, C-6′, C-4‴″, C-4′, C-2‴″, C-1′), 167.79, 170.51, 170.71, 171.19, 171.46 (C-3‴″, C-1′CO, CH₃NCHCO, C-5‴″); FTIR (KBr, cm⁻¹) 2254 (C≡N); Anal. C_{22}H_{23}N₅O₃S (437.51 g/mol) calcd C 60.39, H 5.30, N 16.01; found C 60.76, H 6.09, N 14.64. LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) tᵣ = 19.77, 100% purity, m/z = 438.4 ([M + H]⁺).
3. EXPERIMENTAL SECTION

3.3.39. PREPARATION OF 84

4-Benzylxocarbonyl-5-isobutyl-1,2-dimethyl-3,6-dioxo-1,2,4-triazinane. Compound 81 (5.00 g, 11.2 mmol) was cooled to -25 °C. *N*-methylmorpholine (1.24 g, 12.3 mmol) and isobutyl chloroformate (1.68 g, 12.3 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (7.45 g, 56.0 mmol) was suspended in H₂O (15 mL), and 10 N NaOH (12.0 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of *N*-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min and stirred overnight at room temperature. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with H₂O (30 mL), sat. NaHCO₃ (2 × 30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and evaporated to obtain 82 as a colourless oil without further purification (3.43 g, 100%). Compound 82 (3.43 g, 11.2 mmol) was dissolved in MeCN (20 mL). The resulting solution was treated with DMAP (0.07 g, 0.57 mmol), and (Boc)₂O (3.67 g, 16.8 mmol). It was stirred for 38 h at room temperature. The solvent was removed under reduced pressure. The oily residue was suspended in H₂O (30 mL), a pH of ~ 2 was adjusted (10% KHSO₄), and it was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL), H₂O (30 mL), sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and removed in vacuo. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 84 as a white solid (2.43 g, 65% from 82). mp 81–82 °C; [α]D²⁰ = -8.29 (c = 2.17, CHCl₃); ¹H NMR (500 MHz, DMSO-d₆) δ 0.88–0.90 (m, 6H, CH(CH₃)₂), 1.34–1.49 (m, 2H, CHCH₃), 1.54–1.62 (m, 1H, CH(CH₃)₂), 3.13 (s, 3H, NCH₃), 3.21 (s, 3H, NCH₃), 4.59 (dd, 3J = 8.8 Hz, 3J = 6.9 Hz, 1H, NCHCO), 5.18 (d, 1H, 2J = 12.6 Hz, OCHH), 5.25 (d, 1H, 2J = 12.6 Hz, OCHH), 7.31–7.42 (m, 5H, H_arom); ¹³C NMR (125 MHz, DMSO-d₆) δ 22.06, 22.34, 24.27 (CH₂CHCH₃, CH₃CHCH₃, CH₃CHCH₃), 32.13, 33.76, 38.85 (CHCH₂, 2 × NCH₃), 55.96 (NCHCO), 68.14 (CH₂O), 127.83 (C’-2, C’-6), 128.25 (C’-4), 128.52 (C’-3, C’-5), 135.63 (C’-1), 149.15, 152.22(OCON, NCON), 165.67 (NCHCO); Anal. C₁₇H₂₅N₃O₄ (333.38 g/mol) calcd C 61.25, H 6.95, N 12.60; found C 61.30, H 6.63, N 11.87. MS (ESI) m/z 334.3 ([M + H]⁺).
3.3.40. PREPARATION OF 86

\[
\begin{align*}
\text{N-(Benzyloxycarbonyl)-N-methyleucine 1,2-dimethylhydrazide.} & \quad \text{Compound 85 (5.00 g, 17.9 mmol) was dissolved in dry THF (40 mL) and cooled to -25 °C. To the stirred solution,} \\
& \quad \text{N-methylmorpholine (2.17 mL, 19.7 mmol) and isobutyl chloroformate (2.57 mL, 19.7 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (11.9 g, 89.5 mmol) was} \\
& \quad \text{suspended in H}_2\text{O (5 mL), and 10 N NaOH (18.0 mL) was added under ice-cooling. This} \\
& \quad \text{solution was given to the reaction mixture when the precipitation of N-methylmorpholine} \\
& \quad \text{hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and} \\
& \quad \text{stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was} \\
& \quad \text{extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with} \\
& \quad \text{H}_2\text{O (20 mL), sat. NaHCO}_3\text{(2 × 20 mL), H}_2\text{O (20 mL), and brine (20 mL). The solvent was dried} \\
& \quad \text{(Na}_2\text{SO}_4) \text{and evaporated. The crude product was purified by column chromatography using} \\
& \quad \text{ethyl acetate/petroleum ether (1:1) to obtain 86 as an oily product (3.10 g, 54%).} \\
& \quad \text{^1H NMR} \\
& \quad \text{(500 MHz, DMSO-}\text{d}_6) \text{mixture of s-cis and s-trans isomers } \delta \ 0.82_a, 0.86_b, 0.89_c \ (d_a,} \\
& \quad 3J = 6.0 \text{ Hz, } d_b, 3J = 6.0 \text{ Hz, } d_c, 3J = 6.0 \text{ Hz, } 6H, \text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3), 1.39–1.47 (m, 2H,} \\
& \quad \text{CHCH}_2), 1.53–1.59 (m, 1H, \text{CH}_3\text{CHCH}_3), 2.26_a, 2.43_b \ (d_a, 3J = 5.4 \text{ Hz, } d_b, 3J = 5.7 \text{ Hz, } 3H,} \\
& \quad \text{NHCH}_3), 2.82_a, 2.86_b (2 \times s, 3H, \text{OCONCH}_3), 2.89_a, 2.90_b (2 \times s, 3H, \text{CONCH}_3), 4.74–4.82 \ \\
& \quad (m, 1H, \text{NHCH}_3), 5.02–5.11 (m, 2H, \text{CH}_2\text{O}), 5.43_a, 5.52_b (dd_a, 3J = 10.3 \text{ Hz, } 3J = 4.0 \text{ Hz, dd_b,}} \\
& \quad 3J = 10.6 \text{ Hz, } 3J = 3.9 \text{ Hz, } 1H, \text{CH}_3\text{NCHCO}), 7.28–7.37 (m, 5H, H_{atom});} \\
& \quad ^{13}\text{C NMR (125 MHz, DMSO-}\text{d}_6) \text{mixture of s-cis and s-trans isomers } \delta \ 21.28, 21.36, 23.15, 23.21, 24.72, 24.85 \ \\
& \quad (\text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3, \text{CH}_3\text{CHCH}_3), 29.95, 30.19, 30.87 (\text{OCONCH}_3, \text{NHCH}_3), 34.90,} \\
& \quad 35.03, 37.38, 37.62 (\text{CONCH}_3, \text{CHCH}_2), 52.22, 52.32 (\text{CH}_3\text{NCHCO}), 66.24, 66.44 (\text{CH}_2\text{O,}} \\
& \quad 127.47, 127.60 (C-2', C-6'), 127.84 (C-4'), 128.38, 128.47 (C-3', C-5'), 137.00, 137.21 \ \\
& \quad (C-1'), 155.83, 156.08 (\text{OCON}), 173.09, 173.52 (\text{CH}_3\text{NCHCO}).} \end{align*}
\]
3.3.41. PREPARATION OF 89

*N*-{4-[(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl}-leucine 1,2-dimethylhydrazide.

Compound 81 (10.0 g, 37.7 mmol) was dissolved in dry THF (60 mL). The resulting solution was cooled to -25 °C. *N*-methylmorpholine (4.55 mL, 41.4 mmol) and isobutyl chloroformate (5.40 mL, 41.4 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (10.0 g, 75.2 mmol) was suspended in H₂O (15 mL), and 10 N NaOH (15.0 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of *N*-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at rt. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with H₂O (30 mL), sat. NaHCO₃ (2 × 30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was purified on silica gel using ethyl acetate/petroleum ether (1:1) to obtain 82 as a colourless oil (8.30 g, 72%).

Compound 82 (2.00 g, 6.51 mmol) was dissolved in MeOH (10 mL). The resulting solution was treated with Pd/C (0.20 g) and hydrogenated in a hydrogen flow for 2 h (2 bar, room temperature). Pd/C was filtered off, and MeOH was removed under reduced pressure to obtain 87 without purification (1.30 g). Compound 69 (1.11 g, 4.08 mmol) was suspended in dry CH₂Cl₂ (50 mL) and treated with DMAP (0.04 g, 0.33 mmol). EDC (1.27 mL, 7.17 mmol) was added (the reaction mixture became clear), and the resulting solution was stirred for 15 min at room temperature. Compound 87 (1.30 g) in dry CH₂Cl₂ (10 mL) was added to the reaction mixture. It was stirred for 18 h at room temperature. CH₂CH₂ was removed. The resulting oily residue was suspended in H₂O and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with sat. NaHCO₃ (2 × 50 mL), H₂O (50 mL), and brine (50 mL). The solvent was dried (Na₂SO₄) and evaporated. The oily residue was purified by column chromatography on silica gel using MeOH/CH₂Cl₂ (1:40) as eluent to obtain 89 as a white solid (0.77 g, 44% from 69). mp 68–70 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 0.90 (d, ³J = 1.9 Hz, 3H, CH₃CHCH₃), 0.92 (d, ³J = 1.9 Hz, 3H, CH₃CHCH₃), 1.41–1.47 (m, 1H, CH₃CHCH₃), 1.65–1.76 (m, 2H, CH₂CH₂), 2.55 (d, ³J = 5.4 Hz, 3H, NHCH₃), 2.96 (s, 3H, CONCH₃), 4.89 (q, ³J = 5.6 Hz, NHCH₃), 5.42–5.46 (m, 1H, NHCHCO), 7.37 (dd, ³J = 5.0 Hz, ³J = 3.8 Hz, 1H, H-4’’’), 8.07 (d, ³J = 8.5 Hz, 2H, H-3’, H-5’), 8.10, 8.12–8.13
(dd, $^3J = 3.8$ Hz, $^4J = 1.3$ Hz, 1H, m, 3H, C-3'''', C-2', C-6', C-5'''), 8.48 (d, $^3J = 8.5$ Hz, 1H, NHCHCO); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 21.30, 23.46, 24.86 (CH$_3$CHCH$_3$, CH$_3$CHCH$_3$, CH$_3$CHCH$_3$), 31.06 (NHCH$_3$), 35.05 (CONCH$_3$), 48.28 (NHCHCO), 124.54 (C-5'''), 127.10 (C-3', C-5'), 128.28 (C-3'''), 128.52 (C-2', C-6'), 129.43, 133.11, 134.38, 137.29 (C-4''', C-2''', C-4', C-1'), 165.49, 167.83, 171.47, 174.15 (C-3'', C-1'CO, NHCHCO, C-5'''); Anal. C$_{21}$H$_{25}$N$_5$O$_3$S (427.52 g/mol) calcd C 59.00, H 5.89, N 16.38; found C 58.12, H 5.90, N 16.01.
3.3.42. PREPARATION OF 90

*N-{[4-([5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl)-N-methyleucine 1,2-dimethylhydrazide. Compound 86 (2.50 g, 7.78 mmol) was dissolved in MeOH (10 mL). The resulting solution was treated with Pd/C (0.25 g) and hydrogenated in a hydrogen flow for 3 h (2 bar, room temperature). Pd/C was filtered off, and MeOH was removed under reduced pressure to obtain 88 without purification (1.61 g). Compound 69 (1.58 g, 5.80 mmol) was suspended in dry CH$_2$Cl$_2$ (50 mL) and treated with DMAP (0.05 g, 0.41 mmol). EDC (1.52 mL, 8.59 mmol) was added while the reaction mixture became clear, and it was stirred for 15 min at room temperature. Compound 88 (1.61 g) was dissolved in dry CH$_2$Cl$_2$ (10 mL) and added to the reaction mixture. It was stirred for 42 h at room temperature. CH$_2$Cl$_2$ was removed, and the oily residue was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with sat. NaHCO$_3$ (2 × 50 mL), H$_2$O (50 mL), and brine (50 mL). The solvent was dried (Na$_2$SO$_4$) and evaporated. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (5:1) as eluent to obtain 90 as a white solid (0.81 g, 32% from 69). mp 58–60 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) mixture of s-cis and s-trans isomers δ 0.72$_a$, 0.75$_b$, (d$_a$, $^3$J = 6.6 Hz, d$_b$, $^3$J = 6.6 Hz, 3H, CH$_3$CHCH$_3$), 0.96 (app. t, $^3$J = 5.8 Hz, 3H, CH$_3$CHCH$_3$), 1.28–1.39 (m, 1H, CH$_3$CHCH$_3$), 1.50–1.76 (m, 2H, CH$_2$CH$_2$), 2.11$_a$, 2.54$_b$ (d$_a$, $^3$J = 5.7 Hz, d$_b$, $^3$J = 5.7 Hz, 3H, NHCH$_3$), 2.90$_a$, 2.92$_b$ (2 × s, 3H, CH$_3$NCHCO), 2.96$_a$, 2.97$_b$ (2 × s, 3H, CONCH$_3$), 4.68 (q, $^3$J = 5.6 Hz, 1H, NHCH$_3$), 4.85–4.91 (m, 1H, CH$_3$NCHCO), 7.37 (dd, $^3$J = 4.7 Hz, $^3$J = 3.8 Hz, 1H, H-4’’’), 7.48$_a$, 7.55$_b$ (d$_a$, $^3$J = 8.2 Hz, d$_b$, $^3$J = 8.2 Hz, 2H, H-3’, H-5’), 8.08–8.12 (m, 4H, H-2’, H-6’, H-3’’, H-5’’’); $^{13}$C NMR (125 MHz, DMSO-$d_6$) mixture of s-cis and s-trans isomers δ 21.60, 21.88, 22.34, 23.28, 24.85, 25.14 (CH$_3$NCHCO, NHCH$_3$), 29.32, 30.92, 31.05, 33.64 (CH$_3$NCHCO, NHCH$_3$), 34.75, 35.17, 37.12, 38.09 (CONCH$_3$, CH$_2$CH$_2$), 50.75, 56.03 (CH$_3$NCHCO), 124.55, 126.56, 126.70, 127.19, 127.42, 127.52, 127.72, 129.42, 133.08, 134.35 (C-5’’’’, C-3’, C-5’, C-3’’’, C-2’, C-6’, C-4’’’, C-4’, C-2’’’), 140.09, 140.18 (C-1’), 167.80, 170.13, 170.65, 171.43, 171.86, 173.11 (C-3’’, C-1’CO, CH$_3$NCHCO, C-5’’’); Anal. C$_{22}$H$_{27}$N$_5$O$_3$S (441.55 g/mol) calcd C 59.84, H 6.16, N 15.86; found C 59.59, H 6.55, N 14.72.
3.3.43. PREPARATION OF 92

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\text{N-}[4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl]homocycloleucyl-methylazaalanine-nitrile. Compound 116 (0.30 g, 0.68 mmol) was suspended in MeOH (30 mL) and treated with NaOAc (0.17 g, 2.07 mmol) and BrCN (0.15 g, 1.42 mmol). The resulting mixture was stirred at room temperature for 22 h. The solvent was evaporated under reduced pressure. The residue was suspended in H\_2O and extracted with ethyl acetate (3 ×30 mL), washed with 10\% KHSO\_4 (30 mL), H\_2O (30 mL), sat. NaHCO\_3 (30 mL), H\_2O (30 mL), and brine (30 mL). The solvent was dried over Na\_2SO\_4 and removed under reduced pressure. The crude solid product was purified by column chromatography using ethyl acetate/petroleum ether 1:1 as eluent to obtain 92 as a white solid (0.12 g, 38%). mp 206 °C; \textsuperscript{1}H NMR (500 MHz, CDCl\_3) δ 1.35–1.40 (m, 1H, H\textsubscript{cyclohexane}), 1.49–1.53 (m, 2H, H\textsubscript{cyclohexane}), 1.68–1.79 (m, 3H, H\textsubscript{cyclohexane}), 1.97–2.02 (m, 2H, H\textsubscript{cyclohexane}), 2.94 (s, 3H, N(CH\_3)CN), 3.22 (s, 3H, CONCH\_3), 6.52 (s, 1H, C-1''CONH), 7.22 (dd, \textsuperscript{3}J = 5.1 Hz, \textsuperscript{3}J = 3.8 Hz, 1H, H-4''''), 7.67 (dd, \textsuperscript{3}J = 5.1 Hz, \textsuperscript{4}J = 1.3 Hz, 1H, H-3'''''), 7.91 (d, \textsuperscript{3}J = 8.5 Hz, 2H, H-3'', H-5'''); 7.96 (dd, \textsuperscript{3}J = 3.8 Hz, \textsuperscript{4}J = 1.3 Hz, 1H, H-5'''''), 8.23 (d, \textsuperscript{3}J = 8.6 Hz, 2H, H-2'', H-6'''); \textsuperscript{13}C NMR (125 MHz, CDCl\_3) δ 21.75 (C-3', C-5'), 25.02 (C-4'), 31.94 (C-2', C-6'), 33.11 (NH(CH\_3)CN), 41.07 (CONCH\_3), 59.50 (C-1'), 114.51 (CN), 125.53 (C-5'''''), 127.53 (C-3'', C-5''), 128.04 (C-2'', C-6'''), 128.60, 130.13 (C-3'''', C-4'''''), 132.15, 132.31 (C-2'''', C-4'''), 136.13 (C-1'''), 165.28, 167.96, 171.73, 173.10 (C-1''CONH, C-3'''', NHCCO, C-5'''''); LC-MS(ESI) (90\% H\textsubscript{2}O to 100\% MeOH in 20 min, then 100\% MeOH to 30 min, DAD 220.0–400.0 nm) \textit{t}_r = 15.37, 97\% purity, m/z = 465.2 ([M + H]^+).
3.3.44. PREPARATION OF 94

Homocycloleucine benzyl ester p-toluenesulfonate. 1-Amino-1-cyclohexanecarboxylic acid 93 (4.00 g, 27.9 mmol) and p-toluenesulfonic acid (6.37 g, 37.0 mmol) were suspended in a mixture of toluene and benzyl alcohol 4:1 (100 mL) and heated to reflux under Dean-Stark conditions for 8 h. Toluene was removed, and the white residue was suspended in the -25 °C cold ethyl acetate/petroleum ether (1:1) mixture. The suspension was filtered off, washed with ethyl acetate and petroleum ether, and dried to obtain 94 as a white solid (11.0 g, 97%). mp 165–167 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.38–1.41 (m, 2H, H$_{cyclohexane}$), 1.52–1.59 (m, 4H, H$_{cyclohexane}$), 1.69–1.74 (m, 2H, H$_{cyclohexane}$), 1.94–2.00 (m, 2H, H$_{cyclohexane}$), 2.28 (s, 3H, C-4''''CH$_3$), 5.25 (s, 2H, OCH$_2$), 7.10–7.12 (m, 2H, H-3''', H-5''''), 7.34–7.42 (m, 5H, H$_{arom}'$), 7.48 (d, 2H, $^3$J = 7.9 Hz, H-2''', H-6''''), 8.43 (s, 3H, NH$_3^+$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 20.32 (C-3', C-5'), 20.92 (C-4''''CH$_3$), 23.92 (C-4'), 31.39 (C-2', C-6'), 58.86 (C-1'), 67.53 (OCH$_2$), 125.65 (C-2'', C-6''), 128.20 (C-2''', C-6''', C-3'', C-5'''), 128.54 (C-4''), 128.69 (C-3'''', C-5''''), 135.35 (C-1'''), 137.80 (C-1''''), 145.81 (C-4'''''), 170.87 (COO); Anal. C$_{21}$H$_{27}$NO$_5$S (405.51 g/mol) calcd C 62.20, H 6.71, N 3.45; found C 62.10, H 6.65, N 3.39.
**3.3.45. PREPARATION OF 95**

*N-(4-Cyanophenylcarbamoyl)homocycloleucine benzyl ester.* Compound 94 (3.00 g, 7.40 mmol) and triethylamine (2.25 g, 22.2 mmol) were dissolved in a dry THF (40 mL) and treated with 4-cyanophenyl isocyanate (1.07 g, 7.42 mmol). The resulting reaction mixture was stirred at room temperature for 1 h. THF was removed, and the residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with saturated NaHCO$_3$ (2 × 30 mL), water (30 mL), 10% KHSO$_4$ (30 mL), H$_2$O (30 mL), and brine (30 mL), dried and evaporated under reduced pressure. The crude product was recrystallized from ethyl acetate to obtain 95 as a white solid (2.60 g, 93%). mp 188–190 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.21–1.28 (m, 1H, H$_{cyclohexane}$), 1.41–1.48 (m, 2H, H$_{cyclohexane}$), 1.55–1.57 (m, 3H, H$_{cyclohexane}$), 1.70–1.76 (m, 2H, H$_{cyclohexane}$), 5.07 (s, 2H, OCH$_2$), 6.77 (s, 1H, NHC-1’), 7.22–7.31 (m, 5H, H$_{arom}$’’), 7.52 (d, 2H, $^3$J = 9.2 Hz, H-3’’, H-5’’’), 7.66 (d, 2H, $^3$J = 8.9 Hz, H-2’’, H-6’’’), 8.95 (s, 1H, C-1’’’NH); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 21.04 (C-3’, C-5’’), 24.90 (C-4’), 32.38 (C-2’, C-6’), 57.95 (C-1’), 65.84 (OCH$_2$), 102.81 (C-4’’’), 117.63 (C-2’’, C-6’’’), 119.50 (CN), 127.74 (C-2’’, C-6’’), 127.94 (C-4’’), 128.36 (C-3’’, C-5’’), 133.34 (C-3’’’, C-5’’’), 136.45 (C-1’’’), 144.64 (C-1’’), 154.00 (NHCONH), 174.21 (COO); Anal. C$_{22}$H$_{23}$N$_3$O$_3$ (377.44 g/mol) calcd C 70.01, H 6.14, N 11.13; found C 70.04, H 6.17, N 11.12.
2-{4-[(2-Thiophenecarbonyl)oxyamidino]phenyl}-2,4-diazaspiro[4.5]decan-1,3-dione. Compound 95 (2.40 g, 6.36 mmol) was dissolved in EtOH (50 mL) and treated with DIPEA (1.64 g, 12.7 mmol) and hydroxylamine hydrochloride (0.88 g, 12.7 mmol). The resulting solution was refluxed for 3 h. EtOH was evaporated, the oily residue was dissolved in MeCN (30 mL) and treated with triethylamine (1.30 g, 12.8 mmol) and 2-thiophencarbonyl chloride (1.86 g, 12.7 mmol). The solution was stirred at room temperature for 1 h. The precipitated product was filtrated off and washed with 10% KHSO₄ (3 × 20 mL), H₂O (20 mL), sat. NaHCO₃ (3 × 20 mL), H₂O (20 mL), and n-hexane (20 mL). The dry product was recrystallised from EtOH/H₂O (10:1) to obtain 97 as a white solid (2.30 g, 88%). mp > 230 °C; ¹H NMR (500 Hz, DMSO-d₆) δ 1.29–1.36 (m, 1H, H₇-cyclohexane), 1.54–1.61 (m, 3H, H₈-cyclohexane), 1.68–1.78 (m, 6H, H₉-cyclohexane), 6.99 (s, 2H, NH₂), 7.25 (dd, ³J = 4.8 Hz, ³J = 3.8 Hz, 1H, H‴⁻⁴), 7.49a, 7.84b (da, 2H, ³J = 8.8 Hz, db, 2H, ³J = 8.9 Hz, H‴⁻³, H‴⁻⁵, H‴⁻², H‴⁻⁶), 7.97a, 8.14b (ddₐ, ³J = 4.9 Hz, ⁴J = 1.1 Hz, 1H, ddₕ, 1H, ³J = 3.8 Hz, ⁴J = 1.3 Hz, H‴⁻³, H‴⁻⁴), 8.99 (s, 1H, CONH); ¹³C NMR (125 MHz, DMSO-d₆) δ 20.93 (C-7′, C-9′), 24.54 (C-8′), 33.49 (C-6′, C-10′), 61.08 (C-5′), 126.58 (C-1′⁻², C-1′⁻⁶), 127.39 (C-3′, C-5′), 128.39 (C-4′), 130.81 (C-4″), 132.78, 133.87, 133.98 (C-3‴, C-5‴′, C-2‴), 134.26 (C-1″), 154.44 (NCONH), 156.62 (COON), 159.76 (N=N), 175.75 (NHCOO); Anal. C₂₀H₂₁N₄O₄S (412.46 g/mol) calcd C 58.24, H 4.89, N 13.58; found C 58.25, H 4.89, N 13.36. MS(ESI) m/z = 413.3 ([M + H]⁺).
3.3.47. PREPARATION OF 98

*N-(Phenylcarbamoyl)-homocycloleucine benzyl ester.* Compound 94 (2.00 g, 4.93 mmol) was dissolved in dry THF (50 mL) and treated with DIPEA (3.19 g, 24.7 mmol). The resulting solution was treated with phenyl isocyanate (0.59 g, 4.95 mmol) and stirred for 1 h at rt. After evaporation of the solvent, the resulting residue was suspended in H2O and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO3 (60 mL), H2O (60 mL), 10% KHSO4 (60 mL), H2O (60 mL), and brine (60 mL). The solvent was dried (Na2SO4) and evaporated to obtain 98 as a white solid (1.36 g, 78%). mp 181–182 °C; 1H NMR (500 MHz, DMSO-d6) δ 1.20–1.28 (m, 1H, Hcyclohexane), 1.42–1.49 (m, 2H, Hcyclohexane), 1.54–1.57 (m, 3H, Hcyclohexane), 1.69–1.75 (m, 2H, Hcyclohexane), 1.95–1.98 (m, 2H, Hcyclohexane), 5.06 (s, 2H, OCH2), 6.52 (s, 1H, NHCCO), 6.89–6.92 (m, 1H, Harom), 7.20–7.25 (m, 5H, Harom), 7.30–7.32 (m, 2H, Harom), 7.34–7.36 (m, 2H, Harom), 8.41 (s, 1H, C-1'''NH); 13C NMR (125 MHz, DMSO-d6) δ 21.07 (C-3', C-5'), 24.97 (C-4'), 32.57 (C-2', C-6'), 57.71 (C-1'), 65.72 (OCH2), 117.68 (C-2'''', C-6'''), 121.33 (C-4'''''), 127.68 (C-2'', C-6'''), 127.85 (C-4'''), 128.33, 128.79 (C-3'''', C-5'''', C-3'', C-5''), 136.55 (C-1'''''), 140.26 (C-1''), 154.55 (NHCONH), 174.53 (COO); Anal. C21H24N2O3 (352.43 g/mol) calcd C 71.57, H 6.86, N 7.95; found C 71.55, H 7.01, N 7.99.
3.3.48. PREPARATION OF 100

2-(Phenyl)-2,4-diazaspiro[4.5]decan-1,3-dione. Compound 98 (0.60 g, 1.70 mmol) was dissolved in MeOH (60 mL) and treated with DIPEA (0.44 g, 3.40 mmol). The resulting reaction solution was treated with 1,2-dimethylhydrazine dihydrochloride (0.45 g, 3.38 mmol) and stirred for 2 d at rt. After evaporation of the solvent, the residue was suspended in H₂O and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H₂O (60 mL) and brine (60 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:2) as eluent to obtain 100 as a white solid (0.30 g, 72%). mp 215–217 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 1.30–1.35 (m, 1H, H<sub>cyclohexane</sub>), 1.53–1.61 (m, 3H, H<sub>cyclohexane</sub>), 1.65–1.76 (m, 6H, H<sub>cyclohexane</sub>), 7.33–7.47 (m, 5H, H<sub>arom</sub>), 8.91 (s, 1H, NHCCO); ¹³C NMR (125 MHz, DMSO-d₆) δ 20.92 (C-7', C-9'), 24.53 (C-8'), 33.49 (C-6', C-10'), 60.99 (C-5'), 126.90 (C-2'', C-6''), 127.81 (C-4''), 128.76 (C-3'', C-5''), 132.31 (C-1''), 154.73 (NHCONH), 175.90 (COO); Anal. C₁₄H₁₆N₂O₂ (244.29 g/mol) calcd C 68.83, H 6.60, N 11.47; found C 11.60, H 6.67, N 68.72.
3.3.49. PREPARATION OF 101

4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]-N-hydroxybenzamide. Compound 69 (1.20 g, 4.41 mmol) was dissolved in dry THF (40 mL). The resulting reaction mixture was cooled to -25 °C, treated with N-methylmorpholine (0.49 g, 4.84 mmol) and isobutyl chloroformate (0.66 g, 4.83 mmol), and stirred for 10 min at -25 °C. Hydroxylamine hydrochloride (1.39 g, 20.0 mmol) and sodium hydroxide (0.88 g, 22.0 mmol) were treated with MeOH (30 mL). The precipitated NaCl was filtered off, and the filtrate was added to the solution of the mixed anhydride. The resulting mixture was slowly warm to the room temperature and stirred at room temperature for 4 h. The solvent was removed under reduced pressure, and the residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 80 mL). The combined organic layers were washed with 10% KHSO$_4$ (50 mL), H$_2$O (50 mL), brine (50 mL), and dried over Na$_2$SO$_4$. The crude product was recrystallized from ethanol/ethyl acetate (1:8) to obtain 101 as a white solid (1.01 g, 80%). mp 190 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) 7.36 (dd, $^3$J = 5.1 Hz, $^3$J = 3.8 Hz, 1H, H-4'''), 7.94 (d, $^3$J = 8.9 Hz, H-3', H-5'), 8.09a 8.11–8.13b (dd, $^3$J = 3.8 Hz, $^4$J = 1.3 Hz, 1H, m$_a$, 3H, H-2', H-6', H-3'''', H-5''''), 9.14 (s, 1H, NHOH), 11.39 (s, 1H, NHOH); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 124.53 (C-5'''), 127.34, 127.98, 128.35, 129.45, 133.13, 134.41, 135.73 (C-3', C-5', C-3'''', C-4'''', C-2', C-6', C-2''', C-4', C-1'), 163.50, 167.78, 171.49 (CONHOH, C-3'', C-5''); Anal. C$_{13}$H$_9$N$_3$O$_3$S (287.29 g/mol) calcd C 54.35, H 3.16, N 14.63; found C 54.35, H 3.15, N 14.47.
3.3.50. PREPARATION OF 102

1,2-Dimethylhydrazine-carbonitrile. 1,2-Dimethylhydrazine dihydrochloride (6.70 g, 50.4 mmol) was dissolved in H₂O (20 mL). 5 N NaOH (20.0 mL, 0.10 mol) was added under ice-cooling. Sodium acetate (8.20 g, 0.10 mol) and cyanogen bromide (3.56 g, 33.6 mmol) were added. The resulting reaction mixture was stirred for 20 h at room temperature. It was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (50 mL) and dried over Na₂SO₄. The solvent was evaporated under reduced pressure. The product was purified by distillation to obtain 102 as a colourless fluid (0.95 g, 33%). bp 90 °C at 24 mbar. ¹H NMR (500 MHz, CDCl₃) δ 2.67 (s, 3H, NHCH₃), 2.96 (s, 3H, N(CH₃)CN), 3.78 (bs, 1H, NHCH₃); ¹³C NMR (125 MHz, CDCl₃) δ 35.38 (NHCH₃), 40.83 (N(CH₃)CN), 116.53 (CN); MS(EI) m/z = 85.1 (M⁺).
3.3.51. PREPARATION OF 103

\[\text{N-}[4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]phenylcarbamoyl]homocycloleucine benzyl ester.}\]

Compound 94 (1.69 g, 4.17 mmol) was dissolved in H₂O and treated with 8 mL of 5 N NaOH solution. The aqueous layer was extracted with ethyl acetate (3 × 30 mL), and the combined organic layers were washed with sat. NaHCO₃ (30 mL), H₂O (30 mL), brine (30 mL), and dried over Na₂SO₄ to obtain homocycloleucine benzyl ester without further purification. DMAP (0.034 g, 0.28 mmol) and EDC (0.44 g, 2.83 mmol) were dissolved in dry THF (30 mL) and slowly treated with compound 101 (0.80 g, 2.78 mmol) in dry THF (50 mL). The resulting reaction mixture was stirred at room temperature for 1 h. Homocycloleucine benzyl ester in dry THF (20 mL) was added, and it was stirred at room temperature for 18 h. The solvent was removed under reduced pressure, and the residue was suspended in H₂O. The aqueous suspension was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with 10% KHSO₄ (2 × 50 mL), H₂O (50 mL), sat. NaHCO₃ (2 × 50 mL), H₂O (50 mL), brine (50 mL) and dried over Na₂SO₄. The crude product was recrystallized from EtOH/ethyl acetate (1:1) to obtain 103 as a white solid (0.90 g, 64%). mp 216 °C; \(^1\)H NMR (500 MHz, DMSO-d₆) \(\delta\) 1.22–1.29 (m, 1H, H₇cyclohexane), 1.43–1.50 (m, 2H, H₈cyclohexane), 1.56–1.58 (m, 3H, H₉cyclohexane), 1.71–1.77 (m, 2H, H₇cyclohexane), 1.98–2.00 (m, 2H, H₈cyclohexane), 5.08 (s, 2H, OCH₂), 6.68 (s, 1H, NHCCO), 7.23–7.26, 7.31–7.33 (m, 5H, H₁arom′′′′), 7.35 (dd, \(^3J = 4.9\) Hz, \(^3J = 3.6\) Hz, 1H, H-4′′′′′′), 7.57 (d, \(^3J = 8.8\) Hz, 2H, H-2′′′′, H-6′′′′), 7.93 (d, \(^3J = 8.9\) Hz, 2H, H-3′′′′, H-5′′′′), 8.05a, 8.09b (dd, \(^3J = 3.8\) Hz, \(^4J = 1.3\) Hz, 1H, dd₉, \(^3J = 4.9\) Hz, \(^4J = 1.1\) Hz, 2H, H-3′′′′′′, H-5′′′′′′), 8.83 (s, 1H, C-1′′′′′′′′′′′′NH); \(^13\)C NMR (125 MHz, DMSO-d₆) \(\delta\) 21.08 (C-3′′′′, C-5′′′′), 24.95 (C-4′′′′), 32.49 (C-2′′′′, C-6′′′′), 57.88 (C-1′′′′), 65.82 (OCH₂), 117.70, 118.49, 124.82, 127.73, 127.92, 128.16, 128.37, 129.36, 132.78, 134.01, 136.51, 143.43, 143.91, C-5′′′′′, C-4′′′′′, C-3′′′′′, C-2′′′′′, C-6′′′′, C-4′′′′′, C-3′′′′′, C-5′′′′′, C-4′′′′′′, C-3′′′′, C-5′′′′, C-2′′′′′, C-1′′′′′, C-1′′′′), 154.22 (NHCONH), 168.07, 170.90, 174.35 (C-3′′′′′′, C-5′′′′′′, NHCCO); Anal. C_{27}H_{26}N_{4}O_{4}S (502.59 g/mol) calcd C 64.52, H 5.21, N 11.15; found C 63.85, H 5.26, N 10.61. MS (ESI) \(m/z = 503.3\) ([M + H]⁺).
N-(tert-Butyloxycarbonyl)homocycloleucine. Compound 93 (2.00 g, 14.0 mmol) was dissolved in 2 N NaOH (10 mL). (Boc)$_2$O (6.11 g, 28.0 mmol) in dioxane (10 mL) was added. The resulting reaction mixture was stirred for 40 h at room temperature. Ethyl acetate (30 mL) was added and the layers were separated. The aqueous layer was acidified with concentrated HCl and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H$_2$O (30 mL) and brine (30 mL), and dried over Na$_2$SO$_4$. The solvent was removed under reduced pressure to obtain 107 as a white solid (1.65 g, 48%). mp 172 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 1.15–1.23 (m, 1H, H$_{cyclohexane}$), 1.36 (s, 9H, C(CH$_3$)$_3$), 1.43–1.49 (m, 5H, H$_{cyclohexane}$), 1.58–1.63 (m, 2H, H$_{cyclohexane}$), 1.88 (bs, 2H, H$_{cyclohexane}$), 6.82 (s, 1H, OCONH) 12.03 (s, 1H, COOH); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 21.16 (C-3’, C-5’), 25.16 (C-4’), 28.39 (C(CH$_3$)$_3$), 32.05 (C-2’, C-6’), 58.00 (C-1’), 77.81 (C(CH$_3$)$_3$), 154.94 (OCO), 176.35 (COOH); Anal. C$_{12}$H$_{21}$NO$_4$ (243.30 g/mol) calcd C 59.24, H 8.70, N 5.76; found C 59.11, H 8.49, N 5.79.
3.3.53. PREPARATION OF 108

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\text{N-(\text{tert-Butyloxycarbonyl})homocycloleucyl-glycine-nitrile. Compound 107 (1.00 g, 4.11 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. To the stirred solution, triethylamine (1.24 g, 12.3 mmol) and isobutyl chloroformate (0.62 g, 4.54 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.74 g, 11.3 mmol) was added to the reaction mixture when the precipitation of triethylamine hydrochloride occurred (ca. 5 min). It was allowed to warm to room temperature within 30 min and stirred for additional 3 h. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with 10% KHSO}_4 (30 mL), H}_2 O (30 mL), sat. NaHCO}_3 (30 mL), H}_2 O (30 mL), and brine (30 mL). The solvent was dried (Na}_2 SO}_4) and evaporated. The crude product was purified by column chromatography using ethyl acetate as eluent to obtain 108 (0.78 g, 67%). mp 164 °C; }^{1} \text{H NMR (500 MHz, DMSO-}d}_6 ) \delta 1.18–1.23 (m, 1H, H}_\text{cyclohexane}), 1.36 (s, 9H, C(CH}_3}_3), 1.45 (bs, 5H, H}_\text{cyclohexane}), 1.60–1.65 (m, 2H, H}_\text{cyclohexane}), 1.84 (bs, 2H, H}_\text{cyclohexane}), 4.03 (d, \text{ }^3 J = 5.7 Hz, 2H, NHCH}_2CN), 6.69 (bs, 1H, OCONH), 8.08 (bs, 1H, CONH); {^13} \text{C NMR (125 MHz, DMSO-}d}_6 ) \delta 20.31 (C-3', C-5'), 24.35 (C-4'), 26.87 (NHCH}_2CN), 27.53 (C(CH}_3}_3), 31.12 (C-2', C-6'), 57.76 (C-1'), 77.42 (C(CH}_3}_3), 117.02 (CN), 153.58 (OCO), 174.69 (CONH); Anal. C}_{14}H_{23}N_3O_3 (281.35 g/mol) calcd C 59.77, H 8.24, N 14.94; found C 59.75, H 8.63, N 14.78.
3. EXPERIMENTAL SECTION

3.3.54. PREPARATION OF 109

Homocycloleucyl-glycine-nitrile. Compound 113 (0.52 g, 3.07 mmol) was dissolved in CH$_2$Cl$_2$ (20 mL) and treated with DIPEA (2.78 g, 21.5 mmol). Aminoacetonitrile monosulfate (0.57 g, 3.70 mmol) was added, and the resulting reaction mixture was refluxed for 1 h. The solvent was removed under reduced pressure. The oily residue was suspended in H$_2$O and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H$_2$O (30 mL), sat. NaHCO$_3$ (30 mL), and brine (30 mL). The solvent was dried over Na$_2$SO$_4$ and removed under reduced pressure. The crude oily product was purified by column chromatography using CH$_2$Cl$_2$/MeOH (9:1) as eluent to obtain 109 as a colorless oil (0.40 g, 72%). $^1$H NMR (500 MHz, CDCl$_3$) δ 1.26–1.31 (m, 1H, H$_{cyclohexane}$), 1.35–1.40 (m, 4H, H$_{cyclohexane}$), 1.46 (s, 2H, NH$_2$), 1.64–1.66 (m, 3H, H$_{cyclohexane}$), 1.90–1.97 (m, 2H, H$_{cyclohexane}$), 4.14 (d, $^3J$ = 6.0 Hz, 2H, NHCH$_2$CN), 8.34 (bs, 1H, NHCH$_2$CN); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 20.96 (C-3’, C-5’), 25.03 (C-4’), 27.26 (NHCH$_2$CN), 34.35 (C-2’, C-6’), 57.46 (C-1’), 116.36 (NHCH$_2$CN), 178.24 (CONH).
3.3.55. PREPARATION OF 111

\[ N\text{-}[4\text{-}[5\text{-}(2\text{-Thienyl})-1,2,4\text{-oxadiazol-3-yl}]\text{benzoyl}]\text{homocycloleucyl-glycine-nitrile} \]

Compound 108 (0.30 g, 1.07 mmol) was dissolved in dry THF (10 mL). Dry methanesulfonic acid (0.72 g, 7.49 mmol) was added. The resulting reaction mixture was stirred for 24 h at room temperature. THF was evaporated under reduced pressure. The residue was dissolved in a small amount of H₂O. The resulting solution was adjusted with 2 N NaOH to a pH of ~12–13, extracted with ethyl acetate (3 × 30 mL), washed with brine (30 mL), and dried over Na₂SO₄. The solvent was removed under reduced pressure to obtain homocycloleucyl-glycine-nitrile (109, 0.20 g) as a colorless oil without purification. Compound 69 (0.29 g, 1.07 mmol) was dissolved in dry CH₂Cl₂ (20 mL) and treated with oxalyl chloride (0.19 g, 1.50 mmol) and DMF (0.5 mL). The reaction mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. The resulting residue was dissolved in dry THF (10 mL) and added to a mixture of homocycloleucyl-glycine-nitrile (109, 0.20 g) and DIPEA (0.19 g, 1.47 mmol) in dry THF (10 mL) under ice-cooling. The resulting reaction mixture was stirred for 20 h at room temperature. After evaporation of the solvent, the residue was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL), H₂O (30 mL), sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was purified by recrystallisation from ethyl acetate to obtain 111 (0.09 g, 19% from 108). mp 189 °C; ¹H NMR (500 MHz, DMSO-\text{d}_6) \( \delta \) 1.26–1.30 (m, 1H, H\text{-cyclohexane}), 1.55 (bs, 5H, H\text{-cyclohexane}), 1.75–1.81 (m, 2H, H\text{-cyclohexane}), 2.13–2.15 (m, 2H, H\text{-cyclohexane}), 4.06 (d, \( ^3J = 5.7 \text{ Hz} \), 2H, NHCH₂CN), 7.37 (dd, \( ^3J = 5.1 \text{ Hz} \), \( ^3J = 3.8 \text{ Hz} \), 1H, H-4'''''), 8.08 (d, \( ^3J = 8.9 \text{ Hz} \), 2H, H-3''', H-5''''), 8.10 (dd, \( ^3J = 3.8 \text{ Hz} \), \( ^4J = 1.3 \text{ Hz} \), 1H, H-3'''''), 8.12–8.15 (m, 3H, H-5'''', H-2'', H-6'''), 8.19 (s, 1H, C-1'''CONH), 8.23 (t, \( ^3J = 5.7 \text{ Hz} \), 1H, NHCH₂CN); ¹³C NMR (125 MHz, DMSO-\text{d}_6) \( \delta \) 21.38 (C-3', C-5'), 25.19 (C-4'), 27.76 (NHCH₂CN), 31.81 (C-2', C-6'), 59.68 (C-1'), 117.87 (CN), 124.52 (C-5''''), 126.95 (C-3'', C-5''), 128.34 (C-4''''), 128.90 (C-2'', C-6'''), 129.45 (C-3'''''), 133.16, 134.42 (C-2'''', C-4'''), 137.66 (C-1'''), 165.80, 167.84, 171.52, 174.87 (C-1'''CONH, C-3'''', NHCCO, C-5''''); LC-ESI/MS (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.5–400.2 nm) \( t_r = \) 18.93, 97% purity, \( m/z = 436.4 ([M + H]^+) \).
3.3.56. PREPARATION OF 113

**Homocycloleucine-NCA.** Compound 39 (4.20 g, 15.1 mmol) was dissolved in dry CH$_2$Cl$_2$ (100 mL). Oxalyl chloride (2.88 g, 22.7 mmol) and DMF (0.5 mL) were added. The resulting reaction mixture was stirred for 3 h at room temperature. CH$_2$Cl$_2$ was removed under reduced pressure. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:1) on silica gel to obtain 113 as a white solid (2.11 g, 83%). mp 114 °C; $^1$H NMR (500 MHz, DMSO-$_d_6$) $\delta$ 1.29–1.37 (m, 1H, H$_{cyclohexane}$), 1.44–1.54 (m, 3H, H$_{cyclohexane}$), 1.62–1.68 (m, 2H, H$_{cyclohexane}$), 1.71–1.74 (m, 4H, H$_{cyclohexane}$), 9.43 (s, 1H, NH); $^{13}$C NMR (125 MHz, DMSO-$_d_6$) $\delta$ 20.81 (C-3’, C-5’), 24.21 (C-4’), 33.42 (C-2’, C-6’), 62.42 (C-1’), 150.96 (NHCOO), 173.82 (C-1’COO); MS (ESI) $m/z$ = 187.2 ([M + NH$_4]^+$). Anal. C$_8$H$_{11}$NO$_3$ (169.18 g/mol) calcd C 56.80, H 6.55, N 8.28; found C 57.13, H 6.68, N 8.21.
3.3.57. PREPARATION OF 115

1-(Benzyloxycarbonyl)-3,4-dimethyl-1,3,4-triazaspiro[5.5]undecan-2,5-dione. Compound 40 (0.44 g, 1.38 mmol) was dissolved in MeCN (10 mL). The resulting solution was treated with DMAP (8.4 mg, 0.069 mmol) and (Boc)$_2$O (0.45 g, 2.06 mmol). It was stirred for 24 h at room temperature. The solvent was removed under reduced pressure. The oily residue was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 115 (0.34 g, 71%) as a colorless oil. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.27–1.34 (m, 1H, H$_{cyclohexane}$), 1.46–1.51 (m, 1H, H$_{cyclohexane}$), 1.59–1.64 (m, 4H, H$_{cyclohexane}$), 1.71–1.76 (m, 2H, H$_{cyclohexane}$), 1.99–2.05 (m, 2H, H$_{cyclohexane}$), 3.12 (s, 3H, NCH$_3$), 3.20 (s, 3H, NCH$_3$), 5.16 (s, 2H, CH$_2$O), 7.31–7.38 (m, 5H, H$_{arom}$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 22.27 (C-8', C-10'), 24.52 (C-9'), 30.58 (C-7', C-11'), 32.75, 33.93 (2 × NCH$_3$), 61.51 (C-6'), 68.18 (CH$_2$O), 127.95 (C-2'', C-6''), 128.29 (C-4''), 128.53 (C-3'', C-5''), 135.51 (C-1''), 151.93, 152.75 (OCON, NCON), 167.42 (NCCO); MS (ESI) $m/z$ = 346.4 ([M + H]$^+$).
3. EXPERIMENTAL SECTION

3.3.58. PREPARATION OF 116

\[ N\{-[4-[5-(2-Thienyl)-1,2,4-oxadiazol-3-yl]benzoyl\}homocycloleucine 1,2-dimethylhydrazide. \]

Compound 40 (0.75 g, 2.35 mmol) was dissolved in MeOH (30 mL) and treated with Pd/C (0.075 g). The resulting mixture was hydrogenated in a hydrogen flow at 2 bar and room temperature for 4 h. Pd/C was filtered off, and the solvent was removed under reduced pressure to obtain homocycloleucine 1,2-dimethylhydrazide (106, 0.48 g) as a colorless oil which was used without further purification. Compound 69 (0.64 g, 2.35 mmol) was suspended in dry CH₂Cl₂ (20 mL), treated with DMAP (0.015 g, 0.12 mmol) and EDC (0.36 g, 2.32 mmol), and stirred at room temperature for 10 min. Homocycloleucine 1,2-dimethylhydrazide (106, 0.48 g) in dry CH₂Cl₂ (10 mL) was added, and the resulting solution was stirred for 18 h at room temperature. The solvent was evaporated under reduced pressure. The residue was suspended in H₂O, extracted with ethyl acetate (3 × 30 mL), and washed with sat. NaHCO₃ (30 mL), H₂O (30 mL), and brine (30 mL). The solvent was dried over Na₂SO₄ and removed under reduced pressure. The crude oily product was purified by column chromatography using CH₂Cl₂/MeOH 20:1 as eluent to obtain 116 as a white solid (0.38 g, 37% from 40). mp 189 °C; \(^1^H\) NMR (500 MHz, CDCl₃) \(\delta\) 1.29–1.36 (m, 1H, H\(_{cyclohexane}\)), 1.49–1.57 (m, 2H, H\(_{cyclohexane}\)), 1.64–1.72 (m, 5H, H\(_{cyclohexane}\)), 1.93–1.98 (m, 2H, H\(_{cyclohexane}\)), 2.44 (s, 3H, CONCH₃), 3.10 (s, 3H, CONCH₃), 6.42 (s, 1H, C-1′′CONH), 7.22 (dd, \(3^J = 5.1\) Hz, \(3^J = 3.8\) Hz, 1H, H-4''), 7.66 (dd, \(3^J = 5.1\) Hz, \(4^J = 1.3\) Hz, 1H, H-3''), 7.91 (d, \(3^J = 8.5\) Hz, 2H, H-3'', H-5''), 7.96 (dd, \(3^J = 3.6\) Hz, \(4^J = 1.1\) Hz, 1H, H-5''''), 8.20 (d, \(3^J = 8.5\) Hz, 2H, H-2'', H-6'''); \(^{13}\)C NMR (125 MHz, CDCl₃) \(\delta\) 21.84 (C-3', C-5'), 25.35 (C-4'), 32.23 (C-2'', C-6''), 33.54, 35.67 (NHCH₃, CONCH₃), 59.63 (C-1'), 125.59 (C-5'''), 127.46 (C-3'', C-5'''), 127.87 (C-2'', C-6'''), 128.59, 129.56 (C-3''', C-4'''), 132.10, 132.25 (C-2''', C-4'''), 137.29 (C-1''), 164.98, 168.11, 171.67 (C-1''CONH, C-3'''', C-5'''''). Anal. C\(_{22}\)H\(_{25}\)N\(_5\)O\(_3\)S (439.53 g/mol) calcd C 60.12, H 5.73, N 15.93; found C 59.46, H 5.85, N 15.58.
3.3.59. PREPARATION OF 119

*N-[5-(Dimethylamino)naphthalene-1-sulfonyl]homocycloleucyl-glycine-nitrile.* Compound 109 (0.38 g, 2.10 mmol) was dissolved in dry THF (30 mL). Triethylamine (0.32 g, 3.16 mmol) and 5-(Dimethylamino)naphthalene-1-sulfonyl chloride 117 (0.62 g, 2.30 mmol) were added. The resulting reaction solution was stirred for 24 h at room temperature, and heated to reflux for additionally 24 h. The solvent was removed, and the residue was suspended in H₂O. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (30 mL), and dried over (Na₂SO₄). The crude product was purified by column chromatography using ethyl acetate to obtain 119 as a green solid (0.31 g, 36%). mp 79–81 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 0.80–0.87 (m, 2H, H₆cyclohexane), 0.97–1.07 (m, 4H, H₄cyclohexane), 1.57–1.63 (m, 2H, H₄cyclohexane), 1.74–1.77 (m, 2H, H₆cyclohexane), 2.82 (s, 6H, 2 × CH₃), 3.97 (d, ³J = 5.7 Hz, 2H, NHCH₂CN), 7.24 (d, ³J = 7.3 Hz, 1H, H-6’’), 7.55–7.60 (m, 2H, H-7’’, H-3’’), 7.97 (s, 1H, SO₂NH), 8.04 (t, ³J = 5.5 Hz, 1H, NHCH₂CN), 8.08 (dd, ³J = 7.3 Hz, ²J = 1.3 Hz, 1H, H-4’’), 8.36 a, 8.43 b (d, ³J = 8.8 Hz, db, ³J = 8.5 Hz, 2H, H-8’’, H-2’’); ¹³C NMR (125 MHz, DMSO-d₆) δ 20.87 (C-3’, C-5’), 24.59 (C-4’), 27.78 (NHCH₂CN), 32.51 (C-2’, C-6’), 45.18 (N(CH₃)₂), 61.58 (C-1’), 115.05 (C-6’’), 117.55 (CN), 119.35, 123.54, 127.82, 128.04, 128.95, 129.19, 129.50 (C-8’’, C-2’’, C-3’’, C-7’’, C-4’’, C-8a’’, C-4a’’), 138.55, 151.38 (C-1’’, C-5’’), 174.54 (NHCCO); LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 219.7–300.7 nm) tᵣ = 17.67, 97% purity, m/z = 415.3 ([M + H]+).
3. EXPERIMENTAL SECTION

3.3.60. PREPARATION OF 120

*N-*[(7-Diethylamino)-2-oxo-2H-chromene-3-carbonyl]homocycloleucyl-glycine-nitrile. 7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic acid 118 (0.22 g, 0.84 mmol) was fine suspended in dry THF (15 mL). DIPEA (0.54 g, 4.18 mmol), DMAP (0.01 g, 0.08 mmol), and EDC × HCl (0.17 g, 0.89 mmol) were added, and the resulting reaction mixture was stirred for 20 min at room temperature. Compound 109 (0.15 g, 0.83 mmol) in dry THF (10 mL) was added. It was stirred for 22 h at room temperature, and heated additionally to reflux for 11 h. The solvent was removed under reduced pressure, and the resulting brown residue was suspended in H₂O. The aqueous suspension was further extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO₃ (30 mL) and brine (30 mL), and dried over (Na₂SO₄). The crude product was purified by column chromatography using ethyl acetate/petroleum ether (2:1) to obtain 120 as a green solid (70 mg, 20%). mp 248 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 1.14 (t, 3 J = 7.0 Hz, 6H, N(CH₂C₂H₅)₂), 1.21–1.26 (m, 1H, H_cyclohexane), 1.32–1.40 (m, 2H, H_cyclohexane), 1.58–1.60 (m, 3H, H_cyclohexane), 1.70–1.74 (m, 2H, H_cyclohexane), 2.02–2.05 (m, 2H, H_cyclohexane), 3.48 (q, J = 6.9 Hz, 4H, N(CH₂CH₃)₂), 4.01 (d, J = 5.7 Hz, 2H, NHCH₂CN), 6.64 (d, J = 2.2 Hz, 1H, H-8’’), 6.81 (dd, 3 J = 9.1 Hz, 4 J = 2.5 Hz, 1H, H-6’’), 7.67 (d, J = 9.2 Hz, H-5’’), 8.38 (t, 1H, H-8’’), 8.61 (s, 1H, H-4’’), 8.96 (s, 1H, NHCCO); ¹³C NMR (125 MHz, DMSO-d₆) δ 12.45 (N(CH₂CH₃)₂), 21.07 (C-3’, C-5’’), 24.92 (C-4’’), 27.62 (NHCH₂CN), 31.69 (C-2’, C-6’’), 44.45 (N(CH₂CH₃)₂), 58.75 (C-1’’), 95.99 (C-8’’), 103.78, 109.59, 110.45 (C-6’’), 117.76 (CN), 131.80 (C-5’’), 147.71 (C-4’’), 152.74 (C-7’’), 157.44 (C-8a’’), 161.38, 162.51 (C-3’’CO, C-2’’), 174.70 (NHCCO); LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–480.0 nm) tᵣ = 15.56, 100% purity, m/z = 425.3 ([M + H]⁺).
3.3.61. PREPARATION OF 122

![Chemical structure](image)

N-[(7-Diethylamino)-2-oxo-2H-chromene-3-carbonyl]homocycloleucyl-azaalanine-nitrile. Compound 40 (0.30 g, 0.94 mmol) was dissolved in MeOH (30 mL) and treated with Pd/C (0.030 g). The resulting mixture was hydrogenated in a hydrogen flow at 2 bar and room temperature for 4 h. Pd/C was filtered off, and the solvent was removed under reduced pressure to obtain compound 106 (0.11 g) as a colorless oil without further purification.

7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic acid 118 (0.15 g, 0.57 mmol) was fine suspended in dry THF (15 mL). DMAP (7.0 mg, 0.06 mmol), and EDC (0.10 g, 0.64 mmol) were added, and the resulting reaction mixture was stirred for 10 min at room temperature. Compound 106 (0.11 g) was added. It was stirred for 2 d at room temperature. The solvent was removed under reduced pressure, and the resulting brown residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO$_3$ (30 mL) and brine (30 mL), and dried over (Na$_2$SO$_4$). The crude product was purified by column chromatography using CH$_2$Cl$_2$/MeOH (9:1) to obtain compound 121 as a colourless oil (0.10 mg, 25% from 40). Compound 121 (0.10 g, 0.23 mmol) was dissolved in MeOH (20 mL) and treated with NaOAc (0.059 g, 0.72 mmol) and BrCN (0.051 g, 0.48 mmol). The resulting reaction mixture was stirred for 2 d at room temperature. The solvent was evaporated, and the residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with sat. NaHCO$_3$ (30 mL) and brine (30 mL), and dried over (Na$_2$SO$_4$). The crude product was purified by column chromatography using ethyl acetate to obtain 122 as a green solid (20 mg, 19% from 121). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 1.13 (t, $^3$J = 7.1 Hz, 6H, N(CH$_2$CH$_3$)$_2$), 1.22 (bs, 1H, H$_{cyclohexane}$), 1.39–1.46 (m, 2H, H$_{cyclohexane}$), 1.58–1.63 (m, 3H, H$_{cyclohexane}$), 1.69–1.75 (m, 2H, H$_{cyclohexane}$), 2.15–2.18 (m, 2H, H$_{cyclohexane}$), 2.97 (s, 3H, NCH$_3$), 3.08 (s, 3H, NCH$_3$), 3.48 (q, $^3$J = 7.0 Hz, 4H, N(CH$_2$CH$_3$)$_2$), 6.63 (d, $^4$J = 2.2 Hz, 1H, H-8’’), 6.81 (dd, $^3$J = 9.1 Hz, $^4$J = 2.5 Hz, 1H, H-6’’), 7.66 (d, 1H, $^3$J = 9.2 Hz, H-5’’), 8.60 (s, 1H, H-4’’), 9.14 (s, 1H, NHCCO); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 12.44 (N(CH$_2$CH$_3$)$_2$), 21.16 (C-3’, C-5’), 24.76 (C-4’), 31.46 (C-2’, C-6’), 40.61 (CONCH$_3$), 44.50 (N(CH$_2$CH$_3$)$_2$), 58.22 (C-1’), 96.07 (C-8’’), 107.86, 109.34, 110.45 (C-6’’, C-4a’’, C-3’’), 114.79 (CN), 131.79 (C-5’’), 147.81 (C-4’’), 152.75 (C-7’’), 157.44 (C-8a’’),
161.17, 162.54 (C-3\(^\prime\)CO, C-2\(^\prime\)), 172.98 (NHCCO); LC-MS(ESI) (90% H\(_2\)O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–450.0 nm) \(t_r = 15.47\), 95% purity, \(m/z = 454.3\) ([M + H]\(^+\)).
3.3.62. PREPARATION OF 124

**N-(tert-Butyloxy carbonyl)-S-(isobutyl)cysteine methyl ester.** Sodium (0.87 g, 37.8 mmol) was dissolved under ice-cooling in MeOH (30 mL) and given to N-(tert-butyloxy carbonyl)-L-cysteine methyl ester (123, 5.72 g, 24.3 mmol) in MeOH (30 mL). The resulting solution was treated with isobutyl bromide (4.01 g, 29.3 mmol), and heated to reflux for 5 h. MeOH was removed under reduced pressure, and the resulting yellow oily residue was suspended in H₂O. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL), H₂O (30 mL) and brine (30 mL). The solvent was dried over Na₂SO₄ and evaporated. The oily yellow crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:4) to obtain 124 as a colorless oil (3.88 g, 55%); ¹H NMR (500 MHz, DMSO-d₆) δ 0.92 (d, ³J = 6.6 Hz, 6H, CH(CH₃)₂), 1.37 (s, 9H, C(CH₃)₃) 1.71 (sept, ³J = 6.7 Hz, 1H, CH(CH₃)₂), 2.40 (d, ³J = 7.0 Hz, 2H, (CH₃)₂CHCH₂S), 2.71 (dd, ²J = 13.6 Hz, ³J = 8.9 Hz, 1H, NHCHCHH), 2.82 (dd, ²J = 13.7 Hz, ³J = 5.2 Hz, 1H, NHCHCHH), 3.63 (s, 3H, OCH₃), 4.10-4.15 (m, 1H, NHCHCO), 7.22 (d, ³J = 8.2 Hz, 1H, NHCHCO); ¹³C NMR (125 MHz, DMSO-d₆) δ 21.76, 21.83, 28.10 (CH₃CHCH₃, CH₃CHCH₃, CH₃CHCH₃), 28.25 (C(CH₃)₃), 33.33 (NHCHCH₂), 40.76 ((CH₃)₂CHCH₂S), 52.03, 53.89 (OCH₃, NHCHCO), 78.49 (C(CH₃)₃), 155.38 (OCONH), 171.71 (NHCHCO); Analytical HPLC (90% H₂O to 100% MeOH in 15 min, then 100% MeOH to 30 min) tᵣ = 13.94, purity 96%.
3.3.63. PREPARATION OF 125

\[ \text{N-(tert-Butyloxy carbonyl)-S-(isobutyl)cysteinesulfone methyl ester} \]

Compound 124 (3.70 g, 12.7 mmol) was dissolved in dry CH\(_2\)Cl\(_2\) (20 mL). MCPBA (7.84 g (70%), 31.8 mmol) in CH\(_2\)Cl\(_2\) (20 mL) was slowly added. The resulting reaction mixture was stirred for 18 h at room temperature. The solvent was removed under reduced pressure, and the oily residue was suspended in H\(_2\)O. The aqueous suspension was extracted with ethyl acetate (3 x 30 mL). The combined organic layers were washed with sat. NaHCO\(_3\) (30 mL), H\(_2\)O (30 mL) and brine (30 mL). The solvent was dried over Na\(_2\)SO\(_4\) and evaporated. The oily crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:1) to obtain 125 as a white solid (3.60 g, 88%). mp 86 °C; \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 1.01 (d, \(^3J = 2.5\) Hz, 3H, CH\(_3\)CHCH\(_3\)), 1.02 (d, \(^3J = 2.6\) Hz, 3H, CH\(_3\)CHCH\(_3\)), 1.38 (s, 9H, CH(CH\(_3\))\(_3\)), 2.18 (sept, \(^3J = 6.7\) Hz, 1H, CH\(_3\)CHCH\(_3\)), 2.98–3.07 (m, 2H, (CH\(_3\))\(_2\)CHCH\(_2\)S), 3.47–3.48 (m, 2H, NHCHCH\(_2\)S), 3.66 (s, 3H, OCH\(_3\)), 4.47–4.51 (m, 1H, NHCHCO), 7.49 (d, \(^3J = 8.5\) Hz, 1H, NHCHCO); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 22.41, 22.56, 23.06 (CH\(_3\)CHCH\(_3\), CH\(_3\)CHCH\(_3\), CH\(_3\)CHCH\(_3\)), 28.21 (C(CH\(_3\))\(_3\)), 48.84, 52.68, 54.08, 60.24 (NHCHCO, OCH\(_3\), (CH\(_3\))\(_2\)CHCH\(_2\)S, NHCHCH\(_2\)S), 79.06 (C(CH\(_3\))\(_3\)), 155.18 (OCONH), 170.53 (NHCHCO); Anal. C\(_{13}\)H\(_{25}\)NO\(_6\)S (323.41 g/mol) calcd C 48.28, H 7.79, N 4.43; found C 48.14, H 7.56, N 4.14. MS (ESI) \(m/z = 324.4 ([M + H]^+)\).
3.3.64. PREPARATION OF 127

\[ \text{N-(tert-Butyloxycarbonyl)-S-(isobutyl)cysteinylsulfone-glycine-nitrile.} \]

Compound 125 (3.50 g, 10.8 mmol) was dissolved in a THF/H$_2$O mixture (120 mL). Under ice-cooling, LiOH (0.65 g, 27.1 mmol) was added. When LiOH was dissolved, the reaction solution was stirred for 9 h at room temperature. The reaction solution was acidified with concentrated HCl to a pH of ~2 and extracted with ethyl acetate (3 \( \times \) 80 mL). The combined organic layers were washed with H$_2$O (30 mL) and brine (30 mL). The solvent was dried over Na$_2$SO$_4$ and evaporated to obtain 126 as an oily product without further purification. The oily crude product was dissolved in dry THF (50 mL) and cooled to -25 °C. To the stirred solution, \( N \)-methylmorpholine (1.20 g, 11.9 mmol) and isobutyl chloroformate (1.63 g, 11.9 mmol) were added consecutively. Aminoacetonitrile monosulfate (4.59 g, 29.8 mmol) was dissolved in H$_2$O (5 mL), and 10 N NaOH (6.0 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of \( N \)-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min, and stirred overnight at room temperature. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (3 \( \times \) 120 mL). The combined organic layers were washed with 10\% KHSO$_4$ (30 mL), H$_2$O (30 mL), sat. NaHCO$_3$ (2 \( \times \) 30 mL), H$_2$O (30 mL), and brine (30 mL). The solvent was dried (Na$_2$SO$_4$) and evaporated. The crude product was recrystallized from ethyl acetate to obtain 127 as a white solid (1.60 g, 43\% from 125). mp 151 °C; \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d$_6$}) \( \delta \) 1.02 (d, \( ^3J = 6.7 \) Hz, 6H, CH(CH$_3$)$_2$), 1.39 (s, 9H, C(CH$_3$)$_3$), 2.19 (sept, \( ^3J = 6.7 \) Hz, 1H, CH(CH$_3$)$_2$), 2.97–3.07 (m, 2H, (CH$_3$)$_2$CHCHS), 3.38 (dd, \( ^2J = 14.8 \) Hz, \( ^3J = 9.2 \) Hz, 1H, NHCHCHHS), 3.48 (dd, \( ^2J = 14.8 \) Hz, \( ^3J = 3.5 \) Hz, 1H, NHCHCHHS), 4.06–4.16 (m, 1H, NHCH$_2$CN), 4.45–4.49 (m, 1H, NHCHCO), 7.33 (d, \( ^3J = 8.6 \) Hz, 1H, NHCHCO), 8.72 (t, \( ^3J = 4.4 \) Hz, NHCH$_2$CN); \textsuperscript{13}C NMR (125 MHz, DMSO-\textit{d$_6$}) \( \delta \) 22.51, 22.60, 23.01 (CH$_3$CHCH$_3$, CH$_3$CHCH$_3$, CH$_3$CHCH$_3$), 27.69 (NHCH$_2$CN), 28.27 (C(CH$_3$)$_3$), 49.46 (NHCHCO), 54.52 (NHCHCH$_2$S), 60.11 ((CH$_3$)$_2$CHCHS), 79.06 (C(CH$_3$)$_3$), 117.40 (CN), 155.07 (OCONH), 170.22 (NHCHCO); Anal. C$_{14}$H$_{25}$N$_2$O$_5$S (347.43 g/mol) calcd C 48.40, H 7.25, N 12.09; found C 48.19, H 7.14, N 11.88.
3. EXPERIMENTAL SECTION

3.3.65. PREPARATION OF 128

S-(Isobutyl)cysteinylsulfone-glycine-nitrile methanesulfonate. Compound 127 (1.30 g, 3.74 mmol) was dissolved in dry THF (20 mL). Dry methanesulfonic acid (1.46 mL, 22.5 mmol) was added, and the resulting reaction mixture was stirred for 24 h at room temperature. The precipitated product was filtered out, washed with cold ethyl acetate and petroleum ether, and dried to obtain 128 as a white solid (1.20 g, 93%). mp 109–111 °C; $^1$H NMR (500 MHz, DMSO-$_d$6) $\delta$ 1.04 (d, $^3J$ = 6.7 Hz, 6H, CH(CH$_3$)$_2$), 2.22 (sept, $^3J$ = 6.6 Hz, 1H, CH(CH$_3$)$_2$), 2.40 (s, 3H, CH$_3$SO$_3$), 3.14–3.22 (m, 2H, (CH$_3$)$_2$CHCH$_2$S), 3.52 (dd, $^2J$ = 14.7 Hz, $^3J$ = 7.1 Hz, 1H, NHCHCHHS), 3.69 (dd, $^2J$ = 14.8 Hz, $^3J$ = 5.7 Hz, 1H, NHCHCHHS), 4.24–4.25 (m, 2H, NHCH$_2$CN), 4.31–4.34 (m, 1H, NHCHCO), 8.49 (s, 3H, NH$_3^+$), 9.37 (t, $^3J$ = 5.5 Hz, 1H, NHCH$_2$CN); $^{13}$C NMR (125 MHz, DMSO-$_d$6) $\delta$ 22.46, 23.05 (CH(CH$_3$)$_2$, CH(CH$_3$)$_2$), 27.66 (NHCH$_2$CN), 47.28 (NHCHCO), 53.19 (NHCHCH$_2$S), 59.97 ((CH$_3$)$_2$CHCH$_2$S), 116.93 (CN), 166.86 (NH$_3^+$CHCO).
3.3.66. PREPARATION OF 140

\[ \text{N-( tert-Butyloxycarbonyl)-S-(isobutyl)cysteine.} \]

Compound 139 (5.00 g, 41.3 mmol) was dissolved in a 1:1 EtOH/2 N NaOH mixture (82 mL). 1-Bromo-2-methylpropane (6.22 g, 45.4 mmol) and tetrabutylammonium iodide (0.46 g, 1.25 mmol) were added. The resulting reaction mixture was stirred for 3 d at room temperature. (Boc)\textsubscript{2}O (9.91 g, 45.4 mmol) was added, and it was additionally stirred for 1 d at room temperature. EtOH was evaporated under reduced pressure, the aqueous residue was acidified with conc. HCl (pH ~ 1) and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% KHSO\textsubscript{4} (30 mL) and sat. NaCl (30 mL). The solvent was dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated to obtain 140 as an oily yellow product (10.9 g, 95%). \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}_\textsubscript{6}) \( \delta \) 0.92 (d, \( ^3J = 6.7 \) Hz, 6H, \( \text{CH(CH}_3\text{)}_2 \)), 1.37 (s, 9H, C(CH\textsubscript{3})\textsubscript{3}), 1.72 (sept, \( ^3J = 6.7 \) Hz, 1H, CH(CH\textsubscript{3})\textsubscript{2}), 2.39–2.41 (m, 2H, (CH\textsubscript{3})\textsubscript{2}CHCH\textsubscript{2}S), 2.69 (dd, \( ^2J = 13.6 \) Hz, \( ^3J = 9.1 \) Hz, 1H, NHCHCH\textsubscript{2}H), 2.83 (dd, \( ^2J = 13.6 \) Hz, \( ^3J = 4.8 \) Hz, 1H, NHCHCH\textsubscript{H}), 4.01–4.06 (m, 1H, NHCO), 7.01 (d, \( ^3J = 8.2 \) Hz, 1H, NHCHCO), 12.65 (COOH); \textsuperscript{13}C NMR (125 MHz, DMSO-\textit{d}_\textsubscript{6}) \( \delta \) 21.81, 21.90, 28.14 (CH\textsubscript{3}CHCH\textsubscript{3}, CH\textsubscript{3}CHCH\textsubscript{3}, CH\textsubscript{3}CHCH\textsubscript{3}), 28.31 (C(CH\textsubscript{3})\textsubscript{3}), 33.56 (NHCHCH\textsubscript{2}), 40.79 ((CH\textsubscript{3})\textsubscript{2}CHCH\textsubscript{2}S), 53.89 (NHCHCO), 78.31 (C(CH\textsubscript{3})\textsubscript{3}), 155.44 (OCONH), 172.68 (NHCHCO).
3.3.67. PREPARATION OF 142

\((R)-N-(\text{tert}-\text{Butyloxycarbonyl})-S(\text{isobutyl})\text{cysteinylsulfone}-\text{glycine-nitrile}\). Compound 140 (10.8 g, 38.9 mmol) was dissolved in \(\text{AcOH}\) (80 mL). \(\text{KMnO}_4\) (12.3 g, 77.8 mmol) was dissolved in \(\text{H}_2\text{O}\) (130 mL) and slowly added to the reaction mixture. It was stirred for 2.5 h, followed by the addition of sat. \(\text{KHSO}_3\) solution until the reaction mixture becomes colorless. It was concentrated under reduced pressure, and the aqueous suspension was extracted with ethyl acetate (3 \(\times\) 100 mL). The combined organic layers were washed with \(\text{H}_2\text{O}\) (30 mL), brine (30 mL) and dried over \(\text{Na}_2\text{SO}_4\). The solvent was evaporated to obtain 141 as a colourless oily product (11.1 g, 92%). Compound 141 (2.00 g, 6.46 mmol) was dissolved in dry THF (40 mL), and it was cooled to -25 °C. \(N\)-Methylmorpholine (0.72 g, 7.12 mmol) and isobutyl chloroformate (0.97 g, 7.10 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.47 g, 9.54 mmol) was dissolved in \(\text{H}_2\text{O}\) (2 mL), treated with 2 N \(\text{NaOH}\) (5 mL) and added to the reaction mixture when the precipitation of \(N\)-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. THF was evaporated, and the resulting aqueous suspension was extracted with ethyl acetate (3 \(\times\) 30 mL). The combined organic layers were washed with 10 % \(\text{KHSO}_4\) (30 mL), sat. \(\text{NaHCO}_3\) (30 mL), \(\text{H}_2\text{O}\) (30 mL) and sat. \(\text{NaCl}\) (30 mL). The solvent was dried (\(\text{Na}_2\text{SO}_4\)) and evaporated. The product was recrystallized from ethyl acetate to obtain 142 as a white solid (0.94 g, 42% from 141). mp 162–166 °C; \(^1\text{H NMR}\) (500 MHz, DMSO-\(d_6\)) \(\delta\) 1.02 (d, \(^3\text{J}=6.7\) Hz, 6H, \(\text{CH}(\text{CH}_3)_2\)), 1.39 (s, 9H, C(\(\text{CH}_3)_3\)), 2.56 (sept, \(^3\text{J}=6.7\) Hz, 1H, \(\text{CH}(\text{CH}_3)_2\)), 2.97–3.07 (m, 2H, (\(\text{CH}_3\))\(_2\)\(\text{CHCH}_2\text{S}\)), 3.37 (dd, \(^2\text{J}=14.7\) Hz, \(^3\text{J}=9.3\) Hz, 1H, \(\text{NHCHCHHS}\)), 3.46 (dd, \(^2\text{J}=14.9\) Hz, \(^3\text{J}=3.5\) Hz, 1H, \(\text{NHCHCHHS}\)), 3.46–4.16 (m, 1H, \(\text{NHCH}_2\text{CN}\)), 4.45–4.49 (m, 1H, \(\text{NHCHCO}\)), 7.33 (d, \(^3\text{J}=8.5\) Hz, 1H, \(\text{NHCHCO}\)), 8.71 (bs, \(\text{NHCH}_2\text{CN}\)); \(^{13}\text{NMR}\) (125 MHz, DMSO-\(d_6\)) \(\delta\) 22.50, 22.59, 23.01 (\(\text{CH}_3\)\(\text{CHCH}_3\), \(\text{CH}_3\)\(\text{CHCH}_3\), \(\text{CH}_3\)\(\text{CHCH}_3\)), 27.69 (\(\text{NHCH}_2\text{CN}\)), 28.27 (C(\(\text{CH}_3)_3\)), 49.44 (\(\text{NHCHCO}\)), 54.50 (\(\text{CHCH}_2\)), 60.10 (\(\text{CH}_2\text{S}\)), 79.05 (C(\(\text{CH}_3)_3\)), 117.40 (CN), 155.07 (OCONH), 170.21 (NHCHCO). Anal. C\(_{14}\)H\(_{25}\)N\(_3\)O\(_5\)S (347.43 g/mol) calcd C 48.40, H 7.25, N 12.09; found C 48.32, H 7.18, N 11.83.
3.3.68. PREPARATION OF 144

(R)-N-(Benzoyl)-S-(isobutyl)cysteinylsulfone-glycine-nitrile. Compound 142 (0.68 g, 1.96 mmol) was dissolved in dry THF (10 mL). Under ice-cooling, dry methanesulfonic acid (1.14 g, 11.9 mmol) was added, and the resulting reaction mixture was stirred for 14 h at room temperature. The precipitated product was filtered off, washed with petroleum ether, and dried to obtain compound 143 as a white solid without further purification (0.33 g). Compound 143 (0.33 g, 0.96 mmol) was dissolved in dry THF (30 mL). DIPEA (0.35 g, 2.71 mmol) and benzoyl chloride (0.19 g, 1.35 mmol) were added consecutively. The reaction solution was stirred for 24 h at room temperature. The solvent was evaporated, and the resulting white solid was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% KHSO₄ (30 mL) and brine (30 mL). The solvent was dried (Na₂SO₄) and evaporated. The crude product was recrystallized from ethyl acetate to obtain compound 144 as a white solid (0.28 g, 83%). 

mp 188–192 °C; [α]²₀_D = -54.0 (c = 0.82, THF); ¹H NMR (500 MHz, DMSO-d₆) δ 0.97 (d, ³J = 6.7 Hz, 3H, CH₃CHCH₃), 0.99 (d, ³J = 6.6 Hz, 3H, CH₃CHCH₃), 2.18 (sept, ³J = 6.7 Hz, 1H, CH₃CHCH₃), 3.02–3.10 (m, 2H, (CH₃)₂CHCH₂S), 3.61 (dd, ²J = 14.7 Hz, ³J = 9.3 Hz, 1H, NHCHCHHS), 3.67 (dd, ²J = 14.5 Hz, ³J = 3.5 Hz, 1H, NHCHCHHS), 4.08–4.17 (m, 2H, NHCH₂CN), 4.97–5.01 (m, 1H, NHCHCO), 7.48–7.90 (m, 5H, H_arom), 8.79 (t, ³J = 5.7 Hz, 1H, NHCH₂CN), 8.94 (d, ³J = 8.2 Hz, 1H, NHCHCO); ¹³C NMR (125 MHz, DMSO-d₆) δ 22.44, 22.50, 23.08 (CH₂CHCH₃, CH₂CHCH₃, CH₂CHCH₃), 27.76 (NHCH₂CN), 48.33 (NHCHCO), 53.99 (NHCH₂S), 60.13 ((CH₃)₂CHCH₂S), 117.41 (CN), 127.75 (C-2’, C-6’), 128.38 (C-3’, C-5’), 131.80 (C-4’), 133.71 (C-1’), 166.45 (C-1’CONH), 169.92 (NHCHCO). LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) tᵣ = 10.94, 99% purity, m/z = 352.1 ([M + H]⁺).
3. EXPERIMENTAL SECTION

3.3.69. PREPARATION OF 145

![Chemical structure](image)

**N-(tert-Butyloxy carbonyl)-S-(isobutyl)cysteinesulfone 1,2-dimethylhydrazide.** Compound 141 (2.00 g, 6.46 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. N-methylmorpholine (0.72 g, 7.12 mmol) and isobutyl chloroformate (0.97 g, 7.10 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (1.72 g, 12.9 mmol) was dissolved in H₂O (2 mL), treated with 2 N NaOH (13 mL) and added to the reaction mixture when the precipitation of N-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. The solvent was evaporated, and the resulting white solid was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10 % KHSO₄ (30 mL) and brine (30 mL). The solvent was dried (Na₂SO₄) and evaporated. The oily product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (5:1) as eluent to obtain 145 as a white solid (0.77 g, 34%).

**¹H NMR (500 MHz, DMSO-d₆)**  δ 1.02 (d, ³J = 6.6 Hz, 6H, CH(CH₃)₂), 1.36 (s, 9H, C(CH₃)₃), 2.21 (sept, ³J = 6.7 Hz, 1H, CH(CH₃)₂), 2.48 (d, ³J = 5.7 Hz, 3H, NHCH₃), 2.92–2.97 (m, 4H, CONCH₃, NHCH(CH₃)), 3.01 (dd, ²J =14.0 Hz, ³J =6.5 Hz, 1H, NHCHCH₃), 4.85 (q, ³J = 5.6 Hz, 1H, NHCH₃), 5.21–5.25 (m, 1H, NHCHCO), 6.98 (d, ³J = 8.6 Hz, 1H, NHCHCO).

**¹³C NMR (125 MHz, DMSO-d₆)**  δ 22.56, 22.67, 22.94 (CH₃CHCH₃), 28.30 (C(CH₃)₃), 31.29, 34.90 (2 × NCH₃), 46.69 (NHCHCO), 54.97 (CHCH₂), 60.36 (CH₂S), 78.38 (C(CH₃)₃), 155.02 (OCONH), 170.88 (NHCHCO).
3.3.70. PREPARATION OF 147

\[
\begin{align*}
\text{N-(Benzoyl)-S-(isobutyl)cystinesulfone 1,2-dimethylhydrazide.} & \text{ Under ice-cooling, acetyl chloride (5 mL) was given to dry ethanol (10 mL) and stirred for 10 min at room temperature. Compound 145 (0.70 g, 1.99 mmol) was dissolved in ethyl acetate (10 mL), and the solutions were combined under ice-cooling. It was stirred for 30 min, and the solvent was evaporated. The residue was dissolved in H}_2\text{O, treated with 2 N NaOH to adjust a pH of ~12, and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (30 mL) and dried over Na}_2\text{SO}_4. \text{ The solvent was evaporated to obtain compound 146 (0.20 g, 40%). Compound 146 (0.20 g, 0.80 mmol) was dissolved in dry THF (10 mL). DIPEA (0.21 g, 1.62 mmol) and benzoyl chloride (0.12 g, 0.85 mmol) were added consecutively, and it was stirred for 18 h at room temperature. The solution was evaporated, and it was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% NaHCO}_3 (30 mL) and brine (30 mL), and dried over Na}_2\text{SO}_4. \text{ The solvent was removed under reduced pressure, and the resulting crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (10:1) as eluent to obtain compound 147 as a colourless oil (0.05 g, 18% from 146).} \\
\text{1H NMR (500 MHz, DMSO-}\text{d}_6) & \delta 1.00 (d, \text{ }^3J = 6.6 Hz, 6H, \text{ }\text{CH(\text{CH}_3)_2}), 2.23 (\text{sept, }^3J = 6.7 Hz, 1H, \text{ }\text{CH(\text{CH}_3)_2}), 2.53 (d, \text{ }^3J = 5.7 Hz, 3H, \text{ }\text{NHCH}_3), 2.96 (s, 3H, \text{ CONCH}_3), 3.01 (dd, \text{ }^2J = 14.2 Hz, \text{ }^3J = 6.6 Hz, 1H, \text{ NHCHCHHS}), 3.08 (\text{dd, }^2J = 14.1 Hz, \text{ }^3J = 6.5 Hz, 1H, \text{ NHCHCHHS}), 3.46 (\text{dd, }^2J = 14.5 Hz, \text{ }^3J = 3.5 Hz, 1H, \text{ (CH}_3\text{)}_2\text{CHCHHS}), 3.52 (\text{dd, }^2J = 14.5 Hz, \text{ }^3J = 9.2 Hz, 1H, \text{ (CH}_3\text{)}_2\text{CHCHHS}), 4.93 (q, \text{ }^3J = 5.6 Hz, 1H, \text{ NHCH}_3), 5.72–5.76 (m, 1H, \text{ NHCHCO), 7.46–7.87 (m, 5H, H}_\text{arom}, 8.64 (d, \text{ }^3J = 8.5 Hz, 1H, \text{ NHCHCO),} \text{ }^{13}\text{C NMR (125 MHz, DMSO-}\text{d}_6) & \delta 22.53, 22.56, 23.00 (\text{CH}_2\text{CHCH}_3, \text{ CH}_2\text{CHCH}_3, \text{ CH}_3\text{CHCH}_3), 31.35, 34.91 (2 \times \text{NCH}_3), 45.76 (\text{NHCHCO}), 54.37 (\text{NHCHCH}_2\text{S}), 60.26 (\text{(CH}_3\text{)}_2\text{CHCH}_2\text{S}), 127.51 (\text{C-2', C-6'}), 128.38 (\text{C-3', C-5'}), 131.55 (\text{C-4'}), 134.03 (\text{C-1'}), 166.14 (\text{C-1'CONH}), 170.55 (\text{NHCHCO).}}
\end{align*}
\]
3. EXPERIMENTAL SECTION

3.3.71. PREPARATION OF 148

(R)-N-(Benzoyl)-S-(isobutyl)cysteinylsulfone-methylazaalanine-nitrile. Compound 147 (0.04 g, 0.11 mmol) was dissolved in MeOH (20 mL) and treated with NaOAc (0.03 g, 0.37 mmol) and BrCN (0.02 g, 0.19 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated, and the resulting residue was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (30 mL) and dried over Na₂SO₄. The solvent was removed under reduced pressure, and the resulting crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (2:1) as eluent to obtain 148 as a colourless oil (0.02 g, 48%). [α]₂₀°D = -31.0 (c = 0.51, THF); ¹H NMR (500 MHz, CDCl₃) mixture of s-cis and s-trans isomers δ 1.09–1.14 (m, 6H, CH(CH₃)₂), 2.30–2.43 (m, 1H, CH(CH₃)₂), 2.97–3.04 (m, 2H, NHCH₂S), 3.24, 3.31 (2 × s, 6H, 2 × NCH₃), 3.41–3.47 (m, 2H, (CH₃)₂CHCH₂S), 5.63–5.75 (m, 1H, NHCO), 7.40–7.80 (m, 6H, H₆’-aryl’, NHCHCO); ¹³C NMR (125 MHz, CDCl₃) mixture of s-cis and s-trans isomers δ 22.70, 22.73, 23.69, 23.75, 23.81 (CH₃CHCH₃, CH₃CH₂CH₃, CH₃CHCH₂), 30.77, 30.95, 40.92, 41.07, 45.43, 45.61 (2 × NCH₃, NHCHCO), 53.18, 55.02 (NHCH₂S), 61.92, 62.18 ((CH₃)₂CHCH₂S), 113.49 (CN), 127.25 (C-2’, C-6’), 128.73 (C-3’, C-5’), 132.31 (C-4’), 132.58 (C-1’), 166.91, 167.31 (C-1’S-CONH), 170.26, 170.34 (NHCHCO); LC-MS (ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–400.0 nm) tᵣ = 11.71, 97% purity, m/z = 381.2 ([M + H]⁺).
3.3.72. PREPARATION OF 151

Diethyl phenylsulfonfylmethylyphosphonate. Method A: A mixture of chloromethyl phenyl sulfide (5.00 g, 31.5 mmol) and triethyl phosphate (5.23 g, 31.5 mmol) was heated at 140°C in a sealed tube for 5 h. The resulting solution was distilled under reduced pressure to remove the starting materials. The oily residue was diethyl phenylthiomethylphosphonate 150 (6.78 g, 83%). Diethyl phenylthiomethylphosphonate 150 (3.25 g, 12.5 mmol) was dissolved in dry CH₂Cl₂ and treated with MCPBA (5.39 g, 31.2 mmol). The resulting reaction mixture was stirred at room temperature for 2 d. The precipitated solid was filtrated off, and the filtrate was evaporated under reduced pressure. The resulting residue was purified by column chromatography using ethyl acetate as eluent to obtain 151 as an oily product (1.4 g, 38%).

¹H NMR (500 MHz, DMSO-d₆) δ 1.13–1.16 (m, 6H, 2 × CH₂C₃H₃), 3.92–4.03 (m, 4H, 2 × CH₂CH₃), 4.41 (d, ²JₚH = 17 Hz, 2H, SO₂CH₂), 7.62–7.95 (m, 5H, H-arom); ¹³C NMR (125 MHz, DMSO-d₆) δ 16.06, 16.11 (2 × CH₂CH₃), 51.67 (d, ¹J₁₃C = 94.5 Hz, 62.44, 62.49 (2 × CH₂CH₃), 127.94 (C-2’, C-6’), 129.15 (C-3’, C-5’), 133.92 (C-4’), 140.65 (C-1’).

Diethyl phenylsulfonfylmethylyphosphonate. Method B: A mixture of chloromethyl phenyl sulfide (5.00 g, 31.5 mmol) and triethyl phosphate (5.23 g, 31.5 mmol) was heated at 130°C in a sealed tube for 16 h. The resulting solution was distilled under reduced pressure to remove the starting materials. The oily residue was diethyl phenylthiomethylphosphonate 150 (7.69 g, 94%). Diethyl phenylthiomethylphosphonate 150 (7.68 g, 29.5 mmol) was dissolved in ice acetic acid (20 mL). KMnO₄ (9.34 g, 59.1 mmol) was dissolved in H₂O (50 mL) and dropped slowly to the reaction mixture. It was stirred for 1.5 h at room temperature, followed by the addition of sat. KHSO₃ solution until the reaction mixture becomes colorless. The colorless aqueous suspension was extracted with ethyl acetate (3 × 30 mL), washed with brine (30 mL), and evaporated in vacuo to obtain 151 as an oily colorless product (6.80 g, 79%). ¹H NMR (500 MHz, DMSO-d₆) δ 1.14 (t, ³J = 7.1 Hz, 6H, 2 × CH₂CH₃), 3.93–4.02 (m, 4H, 2 × CH₂CH₃), 4.41 (d, ²JₚH = 17 Hz, 2H, SO₂CH₂), 7.62–7.95 (m, 5H, H-arom); ¹³C NMR (125 MHz, DMSO-d₆) δ 16.07, 16.11 (2 × CH₂CH₃), 51.68 (d, ¹J₁₃C = 131.3 Hz, 62.45, 62.50 (2 × CH₂CH₃), 127.95 (C-2’, C-6’), 129.16 (C-3’, C-5’), 133.92 (C-4’), 140.65 (C-1’).
3.3.73. PREPARATION OF 152

(\textit{E})-1-[(\textit{tert}-Butyloxycarbonyl)amino]-3-(phenylsulfonyl)prop-2-en. Compound 151 (3.20 g, 10.9 mmol) was dissolved in dry THF (20 mL), treated with 60\% NaH in mineral oil (0.52 g, 13.1 mmol), and stirred for 30 min at room temperature. N-Boc-glycinal (1.74 g, 10.9 mmol) was added, and the resulting reaction mixture was stirred for 2 h at rt. THF was evaporated under reduced pressure, and the oily residue was suspended in H$_2$O. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL), washed with brine (30 mL), dried over Na$_2$SO$_4$ and evaporated \textit{in vacuo}. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:1) to obtain 152 as a white solid (1.68 g, 52\%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.35 (s, 9H, C(CH$_3$)$_3$), 3.78 (bs, 2H, CH$_2$NH), 6.65 (d, $^3$J = 15.4 Hz, 1H, CH=CHSO$_2$), 6.82 (dt, $^3$J = 15.1 Hz, $^2$J = 4.6 Hz, 1H, CH=CHSO$_2$), 7.15 (bs, 1H, NHCO), 7.62–7.85 (m, 5H, H$_{arom}$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 28.24 (C(CH$_3$)$_3$), 78.36 (C(CH$_3$)$_3$), 127.25 (C-2', C-6'), 129.71 (C-3', C-5'), 130.00 (CH=CHSO$_2$), 133.76 (C-4'), 140.47 (C-1'), 144.80 (CH=CHSO$_2$), 155.57 (OCONH); MS(ESI) $m/z$ = 315.3 ([M + NH$_4$]$^+$); Anal. C$_{14}$H$_{19}$NO$_4$S (297.37 g/mol) calcd C 56.55, H 6.44, N 4.71; found C 56.47, H 6.38, N 4.75.
3.3.74. PREPARATION OF 153

(E)-1-amino-3-(phenylsulfonyl)prop-2-en hydrochloride. Compound 152 (0.90 g, 3.03 mmol) was dissolved in ethyl acetate (10 mL). AcCl (11.0 g, 0.14 mol) was slowly dropped to MeOH (10 mL) under ice-cooling, and stirred for 10 min. The solutions were combined, and stirred for 10 min at rt. The precipitated white solid was filtered off, and the filtrate was stirred additionally for 30 min at room temperature. The precipitated white solid was again filtered off. The precipitates were combined, washed with n-hexane and dried in vacuo to obtain 153 as a white solid (0.55 g, 78%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 3.71 (dd, $^3$J = 5.4 Hz, $^4$J = 1.6 Hz, 2H, CH$_2$CH=CHSO$_2$), 6.90 (dt, $^3$J = 15.4 Hz, $^4$J = 5.4 Hz, 1H, CH$_2$CH=CHSO$_2$), 7.10 (dt, $^3$J = 15.4 Hz, $^4$J = 1.6 Hz, 1H, CH$_2$CH=CHSO$_2$), 7.65–7.88 (m, 5H, H$_{arom}$), 8.50 (s, 3H, NH$_3^+$); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 38.61 (CH$_2$CH=CHSO$_2$), 127.42 (C-2’, C-6’), 129.84 (C-3’; C-5’), 133.15, 134.08 (C-4’, CH$_2$CH=CHSO$_2$), 138.88, 139.86 (C-1’, CH$_2$CH=CHSO$_2$).
3. EXPERIMENTAL SECTION

3.3.75. PREPARATION OF 155

\[
\begin{align*}
\text{t-BuO} & \text{N} \text{H} \text{H} \text{O} \\
\text{HN} & \text{O} \\
\text{S} & \text{O} \\
2' & 3' & 4' & 5' & 6' \\
1' & \text{1''} & 2'' & 3'' & 4'' & 5'' & 6''
\end{align*}
\]

\text{t-BuO}-\text{N}-\text{(tert-Butyloxycarbonyl)-phenylalanine (E)-3-(phenylsulfonyl)prop-2-en-1-ylamide.}

Compound 154 (1.21 g, 4.56 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. 
\(N\)-methylmorpholine (0.51 g, 5.04 mmol) and isobutyl chloroformate (0.69 g, 5.05 mmol) were added consecutively. Compound 153 (1.07 g, 4.58 mmol) was dissolved in \(H_2O\) (2 mL), treated with 2 N \(NaOH\) (3 mL) and added to the reaction mixture when the precipitation of \(N\)-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 90 min. The solvent was evaporated, and the resulting white solid was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10 % \(KHSO_4\) (30 mL) and brine (30 mL). The solvent was dried (\(Na_2SO_4\)) and evaporated. The oily product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) as eluent to obtain 155 as a white solid (1.05 g, 52% from 154).

\(^1H\) NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 1.28 (s, 9H, \(C(CH_3)_3\)), 2.74 (dd, \(2J = 13.6\) Hz, \(3J = 9.5\) Hz, 2H, \(NHCHCHH\)), 2.90 (dd, \(2J = 13.7\) Hz, \(3J = 5.2\) Hz, 2H, \(NHCHCHH\)) 3.82–4.02 (m, 2H, \(CH_2CH=CHS\)), 4.06–4.11 (m, 1H, \(NHCHCO\)), 6.59 (d, \(3J = 15.1\) Hz, 1H, \(CH_2CH=CHS\)), 6.85 (dt, \(3J = 15.1\) Hz, \(3J = 4.1\) Hz, 1H, \(CH_2CH=CHS\)), 6.99 (d, \(3J = 7.9\) Hz, 1H, \(NHCHCO\)), 7.14–7.24 (m, 5H, \(H_{arom'\prime}\)), 7.62–7.83 (m, 5H, \(H_{arom''\prime}\)), 8.22 (t, \(3J = 5.7\) Hz, 1H, \(NHCH_2CH\)); \(^13C\) NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 28.27 (C(\(CH_3)_3\)), 37.24 (NHCH_2CH), 56.15 (NHCHCO), 78.26 (C(\(CH_3)_3\)), 126.36, 127.19, 128.16, 129.25, 129.69, 129.72, 133.70, 138.15, 140.56, 144.65 (C-4', C-2', C-6', C-2'', C-6'', C-3', C-5', C-3'', C-5'', C-4'', C-1', C-1'', \(CH_2CH=CHS\), \(CH_2CH=CHS\)), 155.48 (OCNH), 171.97 (NHCHCO).
**3.3.76. PREPARATION OF 158**

*N-[4-(5,5-difluoro-7-hydroxy-3-methyl-1-oxo-\((1H,2H)\)-imidazo-[1,5-b]-1-boraisoquinolin-2-yl)butanoyl]phenylalanine (E)-3-(phenylsulfonyl)prop-2-en-1-ylamide.* Compound 155 (1.00 g, 2.25 mmol) was dissolved in ethyl acetate (20 mL). Acetyl chloride (10 mL) was slowly added to MeOH (9 mL) under ice-cooling. The solutions were combined and stirred for 30 min at room temperature. The solvent was evaporated under reduced pressure, the resulting residue was dissolved in H\(_2\)O and adjusted to a pH of ~9 with sat. NaHCO\(_3\). It was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (30 mL). The solvent was dried (Na\(_2\)SO\(_4\)) and evaporated to obtain compound 156 as a colourless oil (0.63 g, 81% from 155). Compound 157 (0.10 g, 0.30 mmol) was dissolved in dry THF (20 mL) and cooled to -25 °C. N-methylmorpholine (30.0 mg, 0.30 mmol) and isobutyl chloroformate (41.0 mg, 0.29 mmol) were added consecutively. Compound 156 (0.19 g, 0.55 mmol) in dry THF (10 mL) was added to the reaction mixture when the precipitation of N-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 3.5 h. The solvent was evaporated, and the resulting white solid was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% KHSO\(_4\) (30 mL), sat. NaHCO\(_3\) (fast with 30 mL), and brine (30 mL). The solvent was dried (Na\(_2\)SO\(_4\)) and evaporated. The brown solid was purified by column chromatography on silica gel using ethyl acetate (twelve 100 mL fractions), then ethyl acetate/MeOH (5:1) as eluent to obtain 158 as a yellow green solid (0.10 g, 52% from 157).

\(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 1.67–1.77 (m, 2H, NCH\(_2\)CH\(_2\)CH\(_2\)CO), 2.08–2.15 (m, 2H, NCH\(_2\)CH\(_2\)CH\(_2\)CO), 2.67 (s, 3H, CH\(_3\)), 2.74 (dd, \(^3\)J = 13.7 Hz, \(^2\)J = 9.3 Hz, 2H, NHCHCH\(_2\)), 2.94 (dd, \(^2\)J = 13.9 Hz, \(^3\)J = 5.7 Hz, 2H, NHCHCH\(_2\)), 3.55–3.67 (m, 2H, NC\(_2\)H\(_2\)CH\(_2\)CH\(_2\)CO), 3.84–3.95 (m, 2H, C\(_2\)H\(_2\)CH=CHS), 4.40–4.44 (m, 1H, NHCHCO), 6.53 (dt, \(^3\)J = 15.1 Hz, \(^4\)J = 1.9 Hz, 1H, CH\(_2\)CH=CHS), 6.73 (dd, \(^3\)J = 8.2 Hz, \(^4\)J = 2.5 Hz, 1H, H-8'''), 6.82 (dt, \(^3\)J = 15.3 Hz, \(^4\)J = 4.2 Hz, 1H, CH\(_2\)CH=CHS), 7.00 (d, \(^4\)J = 2.3 Hz, 1H, H-6'''), 7.12–7.22 (m, 5H, H\(_{\text{arom}}'\)), 7.48 (d, \(^3\)J = 8.5 Hz, 1H, H-9'''), 7.55 (s, 1H, H-10'''), 7.61–7.82 (m, 5H, H\(_{\text{arom}}'\)), 8.27 (t, \(^3\)J = 7.9 Hz, 1H, NHCHCO), (t, \(^3\)J = 5.7 Hz, 1H, NHCHCH\(_2\)); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 12.76 (CH\(_3\)), 14.21 (NCH\(_2\)CH\(_2\)CH\(_2\)CO), 23.87 (NCH\(_2\)CH\(_2\)CH\(_2\)CO), 23.87 (NCH\(_2\)CH\(_2\)CH\(_2\)CO), 24.31 (NCH\(_2\)CH\(_2\)CH\(_2\)CO), 24.31 (NCH\(_2\)CH\(_2\)CH\(_2\)CO), 24.31 (NCH\(_2\)CH\(_2\)CH\(_2\)CO), 24.31 (NCH\(_2\)CH\(_2\)CH\(_2\)CO).
31.97, 37.47, 39.00 (NHCH\textsubscript{2}, NHCH\textsubscript{2}CH, NCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CO), 54.42 (NHCO), 115.34, 118.68 (C-3''', C-5'''), 124.00, 125.02, 126.43, 127.19, 128.19, 129.17, 129.67, 129.79, 133.70, 134.56, 137.89, 140.50, 144.37 (C-4', C-9a''', C-5a''', C-9''', C-2', C-6', C-2'', C-6'', C-3', C-5', C-3'', C-5'', C-4'', C-10'', C-10a''', C-1', C-1'', C-7'''), 161.71, 162.93, 164.18, 171.45 (C-3''', C-1''', NCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CO, NHCH\textsubscript{2}CO). LC-DAD (90\% H\textsubscript{2}O to 100\% MeOH in 20 min, then 100\% MeOH to 30 min, DAD 220.0–500.0 nm) tr = 8.59, 94\% purity. MS(ESI) (pos.) \textit{m/z} = 685.2 ([M + Na]\textsuperscript{+}); MS(ESI) (neg.) 661.2 ([M - H]\textsuperscript{−}); MS(EI) \textit{m/z} = 662.4 (M\textsuperscript{++}).
3.3.77. PREPARATION OF 159

\[ \text{N-} (\text{Benzyloxy carbonyl}) \text{turosyl-glycine-nitrile.} \]

Compound 26 (0.91 g, 2.89 mmol) was dissolved in dry THF (30 mL) and cooled to -25 °C. To the stirred solution, N-methylmorpholine (0.32 g, 3.16 mmol) and isobutyl chloroformate (0.43 g, 3.15 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.11 g, 7.20 mmol) was dissolved in 3 mL H\textsubscript{2}O, treated with 3 mL of 5 N NaOH and added to the reaction mixture when the precipitation of N-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for additional 2 h. The solvent was evaporated under reduced pressure, and the resulting aqueous residue was extracted with ethyl acetate (3 x 60 mL). The combined organic layers were washed with 10% KHSO\textsubscript{4} (30 mL), H\textsubscript{2}O (30 mL), sat. NaHCO\textsubscript{3} (30 mL), H\textsubscript{2}O (30 mL), and brine (30 mL). The solvent was dried (Na\textsubscript{2}SO\textsubscript{4}) and evaporated. The crude product was recrystallized from ethyl acetate/petroleum to obtain 159 as a white solid (0.80 g, 78%). mp 182 °C; H NMR (500 MHz, DMSO-\textsubscript{d}6) \( \delta \) 2.65 (dd, \( ^2J = 13.9 \) Hz, \( ^3J = 10.1 \) Hz, 1H, CHCH\textsubscript{H}), 2.87 (dd, \( ^2J = 13.7 \) Hz, \( ^3J = 4.6 \) Hz, 1H, CHCH\textsubscript{H}), 4.11–4.16 (m, 3H, NHC\textsubscript{H}CN, NHC\textsubscript{H}CO), 4.92 (d, \( ^2J = 12.6 \) Hz, 1H, CHHO), 4.98 (d, \( ^2J = 13.0 \) Hz, 1H, CHHO), 6.64 (d, \( ^3J = 8.5 \) Hz, 2H, H\textsuperscript{-3}, H\textsuperscript{-5}), 7.03 (d, \( ^3J = 8.6 \) Hz, 2H, H\textsuperscript{-2}, H\textsuperscript{-6}), 7.23–7.34 (m, 5H, H\textsuperscript{arom}), 7.51 (d, \( ^3J = 8.5 \) Hz, 1H, NHCHCO), 8.68 (t, \( ^3J = 5.5 \) Hz, 1H NHCH\textsubscript{2}CN), 9.16 (s, 1H, OH); \textsuperscript{13}C NMR (125 MHz, DMSO-\textsubscript{d}6) \( \delta \) 27.24 (NHCH\textsubscript{2}CN), 36.60 (CHCH\textsubscript{2}), 56.50 (NHCHCO), 65.41 (CH\textsubscript{2}O), 115.07 (C-3’, C-5’), 117.59 (CN), 127.55, 127.80, 127.87, 128.41, 130.20, 137.12 (C-2’, C-6’, C-2’’, C-6’’, C-4’’, C-3’’, C-5’’, C-1’, C-1’’), 156.00 (OCONH), 172.42 (CONH); FTIR (KBr, cm\textsuperscript{-1}) 2260 (C≡N); Anal. C\textsubscript{19}H\textsubscript{19}N\textsubscript{3}O\textsubscript{4} (353.37 g/mol) calcd C 64.58, H 5.42, N 11.89; found C 64.79, H 5.69, N 11.26. LC-MS(ESI) (90% H\textsubscript{2}O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–300.0 nm) \( t_r = 14.65 \), 97% purity, \( m/z = 354.4 ([M + H]^+) \).
3.3.78. PREPARATION OF 160

\[ \text{N-(Benzzyloxy carbonyl)-O-(2-fluoroethyl)tyrosyl-glycine-nitrile.} \]

Compound 159 (0.50 g, 1.41 mmol) was dissolved in dry DMF (20 mL) and cooled to \(-10\) °C. The resulting solution was treated with sodium hydride (0.06 g (60 % in mineral oil), 1.50 mmol) and stirred at \(-10\) °C for 30 min. 1-Bromo-2-fluoroethane (0.27 g, 2.13 mmol), which was provided by Reik Löser, was added. The reaction solution was slowly warmed to room temperature and stirred for 19 h. The solvent was evaporated under reduced pressure, the resulting residue was suspended in \(\text{H}_2\text{O}\) and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with \(\text{H}_2\text{O}\) (30 mL) and brine (30 mL). The solvent was dried (\(\text{Na}_2\text{SO}_4\)) and evaporated. The crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1:1) to obtain 160 as a white solid (0.29 g, 51%).

\[ \text{mp 150 °C; } \]

\[ \text{\(\text{^1H NMR (500 MHz, DMSO-d}_6\)) } \delta 2.70 (\text{dd, } J = 13.9 \text{ Hz, } J = 10.4 \text{ Hz, 1H, CHCHH}), 2.92 (\text{dd, } J = 13.9 \text{ Hz, } J = 4.4 \text{ Hz, 1H, CHCHH}), 4.13–4.22 (\text{m, 5H, NHCH}_2\text{CN, NHCHCO, OCH}_2\text{CH}_2\text{F}), 4.71 (\text{dt, } J_{\text{HF}} = 47.7 \text{ Hz, } J = 3.9 \text{ Hz, 2H, OCH}_2\text{CH}_2\text{F}), 4.92 (\text{d, } J = 12.6 \text{ Hz, 1H, CHCO}), 4.97 (\text{d, } J = 12.6 \text{ Hz, 1H, CHCO}), 6.85 (\text{d, } J = 8.9 \text{ Hz, 2H, H-3', H-5'}), 7.17 (\text{d, } J = 8.5 \text{ Hz, 2H, H-2', H-6'}), 7.17–7.34 (\text{m, 5H, H}_\text{arom}), 7.56 (\text{d, } J = 8.5 \text{ Hz, 1H, NHCHCO}), 8.71 (\text{t, } J = 5.4 \text{ Hz, 1H, NHCH}_2\text{CN}); \]

\[ \text{\(\text{\(\text{^13C NMR (125 MHz, DMSO-d}_6\)) \delta 27.23 (\text{NHCH}_2\text{CN), 36.48 (\text{CHCH}_2\), 56.38 (\text{NHCHCO), 65.43 (\text{CH}_2\text{O), 67.10 (d, } J_{\text{CF}} = 18.8 \text{ Hz, OCH}_2\text{CH}_2\text{F), 82.31 (d, } J = 165.6 \text{ Hz, OCH}_2\text{CH}_2\text{F), 114.31 (C-3', C-5'), 117.59 \text{(CN), 127.58, 127.81, 128.40, 130.19, 130.37, 137.08 (C-2', C-6', C-2'', C-6'', C-4'', C-3'', C-5'', C-1', C-1''), 156.01, 156.90 (C-4', OCON), 172.32 (NHCHCO); FTIR (KBr, cm}^{-1} \)) 2247 (\text{C≡N); Anal. C}_{21}\text{H}_{22}\text{FN}_3\text{O}_4 (399.42 g/mol) calcd C 63.15, H 5.55, N 10.52; found C 62.60, H 5.41, N 10.77. LC-MS(ESI) (90% H}_2\text{O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.1–320.7 nm) } t_r = 16.48, 96% purity, m/\text{e} = 404.4 ([\text{M + H}]^+)}. \]
3.3.79. PREPARATION OF 161

**N-(Benzyl oxy carbonyl)-O-(2-fluoroethyl)tyrosyl-methylazaalanine-nitrile.** Compound 38 (1.28 g, 3.35 mmol) was dissolved in dry DMF (20 mL) and cooled to -10 °C. The resulting solution was treated with sodium hydride (0.14 g (60% in mineral oil), 3.50 mmol) and stirred at -10 °C for 30 min. 1-Bromo-2-fluoroethane (0.83 g, (77% purity), 5.03 mmol), which was provided by Reik Löser, was added. The reaction solution was slowly warmed to room temperature, and stirred for 19 h. The solvent was removed under reduced pressure, the resulting residue was suspended in H₂O, and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with H₂O (30 mL) and brine (30 mL), and dried over Na₂SO₄. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:1) as eluent to obtain 161 as a colourless oil (0.53 g, 37%). [α]²⁰D = +22.4 (c = 0.77, CHCl₃); ¹H NMR (500 MHz, DMSO-d₆) mixture of rotamers δ 2.73–2.86 (m, 2H, CHCH₂), 2.98, 3.08, 3.12, 3.22 (4 × s, 6H, 2 × NCH₃), 4.19 (dt, ³J_H,F = 30 Hz, ³J = 3.5 Hz, 2H, OCH₂CH₂F), 4.66–4.77 (m, 3H, OCH₂CH₂F, NHCHCO), 4.95 (s, 2H, CH₂O), 6.88–7.34 (m, 9H, H_arom), 7.78, 7.87 (bs, 3J = 7.9 Hz, 1H, NHCHCO); ¹³C NMR (125 MHz, DMSO-d₆) mixture of rotamers δ 30.28, 30.59, 35.65, 36.04, 40.48, 40.88 (2 × NCH₃, CHCH₂), 52.89, 53.34 (NHCHCO), 65.62 (CH₂O), 67.13 (d, ²J_C,F = 18.8 Hz, OCH₂CH₂F), 82.28 (d, ¹J_C,F = 165.6 Hz, OCH₂CH₂F), 114.21, 114.57, 127.74, 127.91, 128.42, 129.85, 130.28, 130.50, 136.91 (CN, C_arom), 156.28 (OCON), 157.12 (C_aromO), 173.50 (NHCHCO); FTIR (KBr, cm⁻¹) 2222 (C≡N); LC-MS(ESI) (90% H₂O to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 219.7–300.7 nm) tr = 17.23, 96% purity, m/z = 429.5 ([M + H]⁺).
3.4. NMR SPECTRA

Only NMR spectra of not published compounds are shown. For NMR spectra of published compounds, see ‘Supporting Information’ of [192] and [201].
3. EXPERIMENTAL SECTION

3.4.1. NMR SPECTRA OF 84

[Diagram of NMR spectra with chemical shifts and integral values listed below the spectra.]
3. EXPERIMENTAL SECTION

3.4.2. NMR SPECTRA OF 94

![NMR Spectra Image]
3. EXPERIMENTAL SECTION

3.4.3. NMR SPECTRA OF 95
3.4.4. NMR SPECTRA OF 97
3. EXPERIMENTAL SECTION

3.4.5. NMR SPECTRA OF 98
3. EXPERIMENTAL SECTION

3.4.6. NMR SPECTRA OF 100

[Diagram of NMR spectra with various chemical shifts and integrals indicated.]
3.4.7. NMR SPECTRA OF 101

---

**NMR Spectra Data:**

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**1H NMR Spectra Diagram:**

- 8.00 - 7.95 (multiplet)
- 7.37 - 7.36 (singlet)
- 7.92 - 7.93 (singlet)

---

**13C NMR Spectra Diagram:**

- 171.49 (medium downfield)
- 167.78 (high downfield)
- 163.50 (medium downfield)
- 135.73 (medium downfield)
- 134.41 (high downfield)
- 133.13 (medium downfield)
- 129.45 (medium downfield)
- 128.35 (high downfield)
- 127.98 (low downfield)
- 127.34 (medium downfield)
- 124.54 (low downfield)
- 122.04 (low downfield)
---
3. EXPERIMENTAL SECTION

3.4.8. NMR SPECTRA OF 103

[Diagram of NMR spectra with detailed chemical shifts and integrals listed below the graph.]
3.4.9. NMR SPECTRA OF 119
3. EXPERIMENTAL SECTION

3.4.10. NMR SPECTRA OF 120
3.4.11. NMR SPECTRA OF 122
3. EXPERIMENTAL SECTION

3.4.12. NMR SPECTRA OF 124

Integral values and chemical shifts are shown in the figure below. The spectra illustrate the resonance peaks at various ppm values, with integral assignments highlighted in different colors. The data includes peaks at 0.8260, 0.8576, 3.0654, 1.0138, 1.0394, 2.0000, 1.0399, and more, with corresponding integral values such as 0.9233, 0.9101, 7.2251, 7.2087, 4.1483, 4.1369, 4.1313, and others. The chemical shifts range from -0.5 to 9.0 ppm, with specific peaks marked at 2.2351, 2.3067, 2.0000, and so on. The 1H NMR spectrum reveals the detailed resonance pattern of 124.
3. EXPERIMENTAL SECTION

3.4.13. NMR SPECTRA OF 125

[Diagram showing NMR spectra with chemical shifts and integral values listed in ppm]

Chemical Shifts (ppm):
- 7.00 - 7.50
- 2.00 - 2.50
- 0.00 - 0.50

Integral Values:
- 0.7685
- 0.7974
- 2.7931
- 1.8292
- 1.9523
- 0.9587
- 9.0000
- 2.8168
- 2.8212
- 1.0259
- 1.0208
- 1.0126
- 1.0076

[Table showing specific chemical shift values and corresponding integral values]
3. EXPERIMENTAL SECTION

3.4.14. NMR SPECTRA OF 127
3.4.15. NMR SPECTRA OF 128

![NMR Spectra Image]

- **Integral** values and corresponding chemical shifts are listed.
- Peak assignments include:
  - 1.0000 ppm (ppm)
  - 1.0597 ppm
  - 1.1427 ppm
  - 1.9573 ppm
  - 2.2371 ppm
  - 5.7735 ppm
  - 6.3142 ppm
  - 9.3821 ppm
  - 9.3714 ppm
  - 9.3600 ppm

Additional information includes:
- **1H NMR** and **13C NMR** spectra.
- **Chemical Shifts** and **Integrated Areas** for various peaks.
- **Resonance** assignments for **carbon** and **hydrogen** atoms.
3.4.16. NMR SPECTRA OF 140
3.4.17. NMR SPECTRA OF 142

![NMR Spectra Image]

---

integral values

- 8.7155
- 7.3394
- 7.3224
- 4.4857
- 4.4750
- 4.4693
- 4.4580
- 4.1579
- 4.1466
- 4.1233
- 4.1119
- 4.1062
- 4.0949
- 4.0716
- 4.0602
- 3.4942
- 3.4872
- 3.4645
- 3.4576
- 3.3983
- 3.3801
- 3.3693
- 3.3504
- 3.0674
- 3.0548
- 3.0397
- 3.0271
- 3.0119
- 2.9987
- 2.9836
- 2.9703
- 2.2315
- 2.2183
- 2.2051
- 2.1912
- 2.1780
- 2.1647
- 2.1515
- 1.3894
- 1.0263
- 1.0130

(ppm)

- 0.7756
- 0.8063
- 1.0408
- 1.0113
- 1.0344
- 2.0156
- 2.0731
- 8.9965

Integral values

- 6.0000
- 1.0263
- 1.0130

(ppm)

- 1.02
- 3.00
- 3.50
- 4.00

(ppm)

- 3.40
- 7.3

(ppm)

- 4.5
- 7.3

(ppm)

- 3.40
- 7.3

(ppm)

- 4.5
- 7.3

(ppm)

- 3.40
- 7.3

(ppm)

- 4.5
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(ppm)

- 3.40
- 7.3

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- 7.3

(ppm)

- 3.40
- 7.3

(ppm)

- 4.5
- 7.3

(ppm)

- 3.40
- 7.3

(ppm)

- 4.5
- 7.3

(ppm)

- 3.40
- 7.3

(ppm)
3. EXPERIMENTAL SECTION

3.4.18. NMR SPECTRA OF 144
3. EXPERIMENTAL SECTION

3.4.19. NMR SPECTRA OF 145

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3. EXPERIMENTAL SECTION

3.4.20. NMR SPECTRA OF 147
3.4.21. NMR SPECTRA OF 148
3.4.22. NMR SPECTRA OF 151 (method A)
3.4.23. NMR SPECTRA OF 151 (method B)
3.4.24. NMR SPECTRA OF 152

(adapted text and image representation)
3.4.25. NMR SPECTRA OF 153
3. EXPERIMENTAL SECTION

3.4.26. NMR SPECTRA OF 155
3. EXPERIMENTAL SECTION

3.4.27. NMR SPECTRA OF 158

![NMR Spectra Diagram]

- ppm values are listed for various chemical shifts.
3. EXPERIMENTAL SECTION
3.4.28. NMR SPECTRA OF 159
3. EXPERIMENTAL SECTION

3.4.29. NMR SPECTRA OF 160

[Diagram of NMR spectra with peaks and values labeled, including integral values and chemical shifts in ppm.]
3. EXPERIMENTAL SECTION

3.4.30. NMR SPECTRA OF 161

[Image of NMR spectra with chemical shifts and integrals highlighted]
4. REFERENCES


4. REFERENCES


4. REFERENCES


ZUSAMMENFASSUNG


Das primäre Ziel dieser Arbeit war die systematische Entwicklung von Dipeptidnitrilen und Azadipeptidnitrilen als hochpotente und selektive Inhibitoren für die therapeutisch interessanten Cathepsine K und S. Im Rahmen dieser Arbeit wurde außerdem eine fluoreszierende molekulare Sonde für die Detektion und Quantifizierung von Cathepsinen mittels SDS-PAGE etabliert.

Nitril-abgeleitete Dipeptide und Azadipeptide inhibieren Cysteinproteasen durch eine kovalente Interaktion mit dem aktiven Cysteinrest unter reversibler Ausbildung eines
enzymgebundenen Thioimidats bzw. eines enzymgebundenen Isothiosemicarbazids (Abbildung 1).

$X = CH$ (im Fall von Dipeptidnitrilen); $X = N$ (im Fall von Azadipeptidnitrilen).

Durch die stufenweise Optimierung der peptidischen Grundgerüste wurden hochpotente Cathepsin K-Inhibitoren entwickelt, die zusätzlich eine beträchtliche Selektivität für dieses Enzym zeigten. Um die Selektivität für Cathepsin K zu erreichen musste ein großer aromatischer P3-Substituent mit L-Leucin oder Homocycloleucin in der P2-Position kombiniert werden. Im Fall des L-Leucin-abgeleiteten Azadipeptidnitrils war außerdem die Methylierung des P3-P2-Linkers nötig. Generell war die inhibitorische Aktivität der Azadipeptidnitrile höher als die der entsprechenden Kohlenstoff-Analoga (Tabelle 1).


<table>
<thead>
<tr>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$R^3$</th>
<th>$R^4$</th>
<th>$X$</th>
<th>Cath L ($nM$)</th>
<th>Cath S ($nM$)</th>
<th>Cath K ($nM$)</th>
<th>Cath B ($nM$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H$</td>
<td>iso-Butyl</td>
<td>$H$</td>
<td>$H$</td>
<td>$CH_2$</td>
<td>940</td>
<td>140</td>
<td>2,9</td>
<td>$&gt; 22000$</td>
</tr>
<tr>
<td>Me</td>
<td>iso-Butyl</td>
<td>$H$</td>
<td>Me</td>
<td>NMe</td>
<td>2700</td>
<td>140</td>
<td>0,63</td>
<td>510</td>
</tr>
<tr>
<td>$H$</td>
<td>-(CH$_2$)$_5$-</td>
<td>$H$</td>
<td>$CH_2$</td>
<td>$&gt; 5000$</td>
<td>$&gt; 5000$</td>
<td>13</td>
<td>$&gt; 5000$</td>
<td></td>
</tr>
<tr>
<td>$H$</td>
<td>-(CH$_2$)$_5$-</td>
<td>Me</td>
<td>NMe</td>
<td>280</td>
<td>78</td>
<td>0,35</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

S3, S2 – Bindungstaschen von Cathepsin K

Im zweiten Projekt dieser Arbeit wurde die Bindungstasche S3 von Cathepsin S systematisch untersucht. Dafür wurde ein großer P2-Rest mit unterschiedlichen aromatischen P3-Substituenten kombiniert. Die synthetisierten Substanzen wurden an den menschlichen Cathepsinen L, S, K und B evaluiert und zeigten alle inhibitorische Aktivitäten im
nanomolaren Bereich gegenüber Cathepsin S. Obwohl die Bindungstasche S3 von Cathepsin S auch größere aromatische P3-Substituenten akzeptierte, erreichte das Dipeptidnitril mit einem kleinen Benzoylrest in der P3-Position die höchste Hemmaktivität. Weiterhin zeigte dieser Inhibitor auch eine beachtliche Selektivität für Cathepsin S über die Antitargets, Cathepsine L, K und B. Das entsprechende Azadipeptidnitril war weniger selektiv, während seine inhibitorische Aktivität deutlich höher lag (Tabelle 2).

**Tabelle 2.** $K_i$-Werte eines Cathepsin S-selektiven Dipeptidnitrils und seines Stickstoff-Analogons

<table>
<thead>
<tr>
<th>X</th>
<th>Cath L (nM)</th>
<th>Cath S</th>
<th>Cath K (nM)</th>
<th>Cath B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>37000</td>
<td>33</td>
<td>&gt; 40000</td>
<td>24000</td>
</tr>
<tr>
<td>NMe</td>
<td>15</td>
<td>0,55</td>
<td>0,66</td>
<td>5,8</td>
</tr>
</tbody>
</table>

S3, S2 – Bindungstaschen von Cathepsin S

In einem weiteren Projekt wurde eine fluoreszierende molekulare Sonde für die Visualisierung von thiolabhängigen Cathepsinen entwickelt. Diese Sonde besteht aus vier Teilen: (1) einem Vinylsulfon für eine irreversible Interaktion mit dem aktiven Cysteinrest im Sinne einer Michael Addition; (2) L-Phenylalanin in der P2-Position; (3) einem Linker und (4) einem fluoreszierenden Reporter. Die Hemmaktivität der synthetisierten Sonde wurde an Cathepsinen L, S, K and B evaluiert und die grundsätzliche Möglichkeit zur Visualisierung von Cathepsinen wurde am Beispiel von Cathepsin K gezeigt (Abbildung 2).

**Abbildung 2.** Eine fluoreszierende molekulare Sonde.

FG – fluoreszierende Gruppe.
SELBSTSTÄNDIGKEITSERKLÄRUNG


Bonn, Februar 2012

Maxim Frizler
In erster Linie möchte ich meinem Doktorvater, Herrn Professor Dr. Michael Gütschow, für die freundliche Aufnahme in seinem Arbeitskreis und für die Überlassung dieses außerordentlich interessanten Themas danken. Weiterhin danke ich ihm für die fortlaufende Unterstützung der Arbeit, die anregenden Diskussionen und seine stets konstruktiven Ratschläge.

Frau Professor Dr. Christa E. Müller danke ich sehr herzlich für die Übernahme des Koreferates und für die Betreuung im Rahmen der NRW Forschungsschule Biotech-Pharma.

Mein besonderer Dank gilt Frau Friederike Lohr und Frau Janina Schmitz für die überaus wertvolle Mitarbeit auf dem spannenden Gebiet der Cathepsine.

Weiterhin möchte ich unseren Praktikanten, Anne Cöse, Norbert Furtmann, Henriette Günther, Christoph Hauser, Judith Kinzy, Julia Kläs, Patrick Jim Küppers, Michael Lülsdorff und Anna Schulz-Fincke, danken, die wir hoffentlich für die Wissenschaft begeistern konnten.

Für die erfolgreiche Zusammenarbeit danke ich auch ganz herzlich unseren Kooperationspartnern, Santos Fustero, Reik Löser, Vanessa Rodrigo und Ilia V. Yampolsky.


Mein tiefster Dank gilt meinen Eltern und meiner Schwester Julia, die mir immer unterstützend zur Seite standen.

Die Durchführung der Arbeit wurde mir durch ein Stipendium der NRW Forschungsschule Biotech-Pharma ermöglicht.