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EPIGRAPH

Volli,

e volli sempre,

e fortissimamente volli.

(Vittorio Alfieri, 1783)

RESUMO

A doença de Chagas, causada pelo parasito protozoário *Trypanosoma cruzi*, continua sendo um grave problema de saúde devido à terapia inadequada e à falta de uma vacina eficaz. Novos fármacos que sejam seguros e eficazes são, portanto, criticamente necessários. A cruzaina (Cz), principal protease cisteínica (CP) do *T. cruzi*, é uma enzima essencial para o ciclo de vida do parasito e tem sido utilizada como alvo viável para a busca e identificação de novas entidades químicas (NCE) como agentes tripanossomicidas. Alguns resultados recentes demonstram que os inibidores covalentes reversíveis da Cz podem bloquear os passos de diferenciação celular no parasito e levá-lo à morte.

Nesta tese, por emprego de abordagem racional baseada em hipóteses, foram planejados e sintetizados diferentes séries de inibidores da Cz baseadas em dipeptidil nitrilas. Em especial, o esqueleto molecular foi modificado para compreender as interações S3/P3, o papel do grupo ciclopropila no processo de reconhecimento bimolecular e o mapeamento S1/S1' para melhorar a afinidade e avaliar a seletividade frente a outras CPs presentes em células de mamíferos.

A partir do protótipo **Neq0570** ($pK_i = 6,3$), a afinidade para Cz foi aumentada em 1,6 unidades log para o **Neq0922**, $pK_i = 7,9$, por uma combinação de modificações estruturais em P3, P2 e P1.

Dos resultados relevantes obtidos, destacam-se três novos inibidores da Cz (**Neq0877**, **Neq0938** and **Neq0940**) equipotentes ao Benzonidazol – Bz, (o fármaco atualmente usado no Brasil para o tratamento da doença de Chagas) em ensaios *in vitro* para a avaliação da atividade antitripanossomal sobre o *T. cruzi*. Considerando-se que o índice de seletividade (SI) do Bz é > 20 , como no caso desses inibidores, testes *in vivo* serão necessários para avaliar a potência frente à forma aguda da doença de Chagas.

Inibidores bioisotéricos de dipeptidil nitrilas estão sob investigação pré-clínica como agentes tripanossomicidas. Destarte, realizamos a síntese e os estudos de relação estrutura-atividade (SAR) de inibidores da Cz por substituição da amida em P3/P2 por trifluoroetilamina. Foram identificados três novos inibidores de Cz com afinidade em concentração nanomolar de um dígito (**Neq0642**, **Neq0643** e **Neq0659**) com alta seletividade sobre a catepsina L de humanos (CatL). Os efeitos da configuração do

centro estereogênico em P3/P2 associados à substituição de diferentes grupos em P1 levam a fortes efeitos não-aditivos nas SARs.

A troca bioisostérica pelo grupo sulfonamida em P3/P2 resultou em uma nova série de inibidores que foi avaliada em um painel de CPs de agentes infecciosos e de seres humanos. Há uma reconhecida SAR na inibição da Cz, ao mesmo tempo em que revelam uma inibição na ordem nanomolar para a CatS, com elevada seletividade para os outras CPs de mamíferos (CatB, CatK e CatL).

A exploração da SAR também incorpora os efeitos do grupo eletrofílico nitrila por inclusão de “warheads” alternativos. As constantes de inibição representadas por aldeídos e nitrilo-azadipeptídios são duas ordens de grandeza maiores para os melhores inibidores de Cz. Além disso, o inibidor de Cz nitrilo-azadipeptídico (**Neq0690**) foi usado na primeira geração de inibidores da CPB de *Leishmania mexicana* (LmCPB) obtida na forma cristalina com alta resolução da estrutura cristalográfica.

ABSTRACT

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, remains a serious health problem due to inadequate therapy and lack of an effective vaccine. Therefore, new drugs that are safe and efficient are critically needed. Cruzain (Cz), the main *T. cruzi* cysteine protease (CP), is an essential enzyme for the life cycle of the parasite and has been used as a viable target for the search and identification of new chemical entities (NCE) acting as trypanocidal agents. Recent literature results demonstrate that reversible covalent Cz inhibitors are able to block the cell differentiation steps in the parasite and to kill it.

To this end, by applying a knowledge-based design approach, we have designed and synthesized a new series of dipeptidyl nitrile inhibitors of Cz aimed at leveraging the S3/P3 interactions, understand the role of the cyclopropane in the bimolecular recognition process and map the S1/S1' to enhance affinity and selectivity over other CPs. Herein, we report three new Cz inhibitors (**Neq0877**, **Neq0938** and **Neq0940**) being equipotent with Benznidazole (the only current drug used to treat Chagas disease in Brazil) with SI (selective index) ratio over 20 (respect to the host cell) and therefore making them attractive synthetic targets for further *in vivo* testing against the acute form of Chagas disease.

Dipeptidyl nitrile bioisostere inhibitors are under preclinical investigation as trypanocidal agents. Therefore, we performed the synthesis and structure-activity relationship (SAR) investigation of nitrile-based Cz inhibitors incorporating a trifluoroethylamine moiety as P3/P2 amide replacement. Results display three new one-digit nanomolar Cz inhibitors (**Neq0642**, **Neq0643** and **Neq0659**) with high selectivity over CatL. In addition, this work reveals that the effects of configuration at the P3/P2 amide replacement and different substitution in P1 lead to strong nonadditivity outcomes.

The bioisosteric exchange by a sulfonamide moiety on P3/P2 resulted in a new series of inhibitors that was evaluated on a panel of infectious and human CPs. Data displays an interesting flat SAR for Cz while disclosing strong CatS inhibition with high selectivity for other mammalian CPs (CatB, CatK, and CatL).

SAR investigation also includes the effects of the nitrile electrophilic group by including alternative warheads. Inhibition constants represented by aldehydes and nitrile azadipeptides are two orders of magnitude larger for the best Cz inhibitors. Besides, the nitrile azadipeptide Cz inhibitor (**Neq0690**) was used in the first generation of *Leishmania mexicana* CPB (LmCPB) high-resolution x-Ray crystal structure.

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EXORDIUM

This Ph.D. thesis is composed of six main chapters identified with bold Latin numerals (**I** to **VI**). Each chapter represents a scientific article in a peer-reviewed international scientific journal (see list below) where the Ph.D. candidate is the first author or co-author.

List of the main papers presented in this thesis:

Chapter **I**: CIANNI, L.; SARTORI, G.; ROSINI, F.; DE VITA, D.; PIRES, G.; LOPES, B. R.; LEITÃO, A.; BURTOLOSO, A. C. B.; MONTANARI, A. C. Leveraging the cruzain S3 subsite to increase affinity for reversible covalent inhibitors. **Bioorganic Chemistry**, v. 79, p. 285–292, set. 2018.

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Related articles:

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LIST OF ABBREVIATIONS AND ACRONYMS

ACN	<i>Acetonitrile</i>
AcOEt	<i>Ethyl acetate</i>
Bn	<i>Benzyl</i>
Boc	<i>tert-Butoxycarbonyl</i>
Boc-AA-OH	<i>N-tert-Butoxycarbonyl-Amino Acid-carboxyl acid</i>
DCM	<i>Dichloromethane</i>
DDQ	<i>2,3-Dichloro-5,6-dicyano-1,4-benzoquinone</i>
DIPEA	<i>N,N-Diisopropylethylamine</i>
DME	<i>1,2-Dimethoxyethane</i>
DMF	<i>Dimethylformamide</i>
DMSO	<i>Dimethyl sulfoxide</i>
ESI-MS	<i>Electrospray Ionization Mass Spectrometry</i>
FT-IR	<i>Fourier-Transform Infrared Spectroscopy</i>
h	<i>Hour</i>
HATU	<i>(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)</i>
HPLC	<i>High-performance liquid chromatography</i>
HRMS	<i>High Resolution Mass Spectrometry</i>
LC-MS	<i>Liquid Chromatography–Mass Spectrometry</i>
logP	<i>The 10-based logarithm of partition coefficient</i>
Leu	<i>Leucine</i>
LogS	<i>The calculated 10-based logarithm of the solubility</i>
min	<i>Minute</i>
MM	<i>Molecular Mass</i>
MW	<i>Molecular Weight</i>
PDB ID	<i>Protein Databank Identification</i>
Phe	<i>Phenylalanine</i>

PMF	<i>Potential of Mean Force</i>
<i>p</i> -TsCl	<i>4-Toluenesulfonyl chloride</i>
Py	<i>Pyridine</i>
QM/MM	<i>Quantum Mechanics / Molecular Mechanics</i>
rt	<i>Room temperature</i>
TBTU	<i>2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate</i>
TFA	<i>Trifluoroacetic Acid</i>
TFAA	Trifluoroacetic anhydride
THF	<i>Tetrahydrofuran</i>
Thr	<i>Threonine</i>
TLC	<i>Thin Layer Chromatography</i>
TPSA	<i>Topological Polar Surface Area</i>
Trp	<i>Tryptophan</i>
UV	<i>Ultraviolet</i>
UV DAD	<i>Ultraviolet Diode Array Detection</i>

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1. INTRODUCTION

1.1. Chagas disease: general aspects

Chagas disease (CD), which was discovered in 1909 by the Brazilian physician Carlos Chagas (COURA, 2013), is one of the main socio-economic problems faced in Latin American countries. Among the 21 states where this disease is endemic, Bolivia stands out with the highest rates of people infected with the parasite (BERN et al., 2011; JACKSON; PINTO; PETT, 2014; PEREIRA; NAVARRO, 2013). Due to immigration, this disease also affects people in other continents. Currently, cases have been detected in the United States, Canada and some European countries. According to the World Health Organization (WHO), the number of people infected with this parasite is likely to be between 7 and 8 million. Over 10.000 people die annually because of this disease (“Chagas disease (American trypanosomiasis)”).

The etiological agent is the protozoa parasite *Trypanosoma cruzi*, which is transmitted by blood-sucking reduviid bugs of the subfamily Triatominae (PRATA, 2001). Clinically, the CD is characterized by an acute phase that has an average duration of two months, being followed by a chronic stage that typically lasts throughout the life of the host. In most cases, especially in adults, the acute phase of the disease is oligosymptomatic and thereby not being detected by sufferers or by the health agent. The only two existing drugs in the market, Benznidazole and Nifurtimox, show high toxic side effects and inefficiency in the chronic stage of the disease (CASTRO; DEMECCA; BARTEL, 2006; COURA; VIÑAS, 2010). Beyond doubt, new drugs that are safe and efficacious are critically needed. Consequently, initiatives such as Drugs for Neglected Diseases Initiative (DNDi) have led to collaborative efforts worldwide in the discovery of new macromolecular targets (URBINA, 2010) and in the identification of new trypanocidal agents .

1.2. Cruzain (Cz)

Enzymes classified as proteases are responsible for the catalysis of peptide bond hydrolysis reactions. They play an essential role in various pathological processes such as rheumatoid arthritis, cardiovascular diseases, bacterial and viral infections, cancer and Alzheimer's disease (SALISBURY; ELLMAN, 2006). Proteases are also key enzymes in the pathogenicity of many parasitic diseases and are significant targets because they are involved in the survival and replication of the parasites, inducing damage to the host tissue, facilitating the invasion of the parasite or enhancing the use of host protein metabolites. Hence, elucidation/description of the biological function of proteases and acquisition of complete knowledge of biochemistry and structure are requirements for designing antiparasitic drugs that specifically modulate the proteolytic action (MCKERROW, 1989; (RENSLO; MCKERROW, 2006).

Cruzain (Cz), a recombinant form of the enzyme cruzipain (EC 3.4.22.51; CAZZULO et al., 1990; GILLMOR; CRAIK; FLETTERICK, 1997), is the most abundant cysteine protease present in the *T. cruzi*. It is essential for the development and survival of the parasite inside and outside the host cell in all forms of its life cycle, which makes it a desirable target for the development of new chemotherapeutic agents for CD (VIEIRA et al., 2005).

Like other CPs, Cz is small (22.7 kDa), monomeric and composed of two folded and equally sized domains (EAKIN et al., 1993). These domains are divided by the enzyme's active site, which is V-shaped and primarily exposed to solvent. A catalytic triad cysteine-histidine-asparagine forms the active site. A water molecule between cysteine and histidine enables a proton transfer, forming a thiolate-imidazolium ion pair (PAASCHE; SCHIRMEISTER; ENGELS, 2013). The thiolate ion acts as nucleophile on the peptide carbonyl carbon, forming the acyl-enzyme intermediate, which undergoes subsequent hydrolysis.

The substrate binds in the active site cleft in an extended conformation, interacting with residues in both directions from the scissile bond (Figure 1) in between the two domains of the enzyme. The non-primed side refers to the N-terminal part and the primed side to the C-terminal region of the substrate.

Figure 1. Substrate binding to CPs.

The corresponding amino acid residues in position P3, P2, P1, P1', P2', P3' occupy the subsites (specificity pockets) S3, S2, S1, S1', S2', S3'. The bond cleaved by the endopeptidase protease is represented in red. Source: CIANNI, L. et al. Can Cysteine Protease Cross-Class Inhibitors Achieve Selectivity? Can cysteine protease cross-class inhibitors achieve selectivity? **Journal of Medicinal Chemistry**, jul. 2019. <https://doi.org/10.1021/acs.jmedchem.9b00683>.

The three-dimensional structure of the Cz enzyme, shown in Figure 2, consists of a polypeptide chain with 215 amino acid residues enveloped in two domains, one α -helicoidal and the other composed of extensive β -sheets interactions. Residues of the catalytic triad are identified as Cys25, His159 and Asn175.

Several classes of covalent inhibitors effectively modulate Cz as well as other cysteine proteases, although the development of non-covalent inhibitors is still a vital strategy for inhibition of the enzyme. Among covalent inhibitors, there are compounds with reactive groups containing an electrophilic carbon (*warhead*) susceptible to the nucleophilic attack by the activated cysteine of the enzyme (FERREIRA et al., 2009; WIGGERS et al., 2013).

Figure 2- (A) Representation of the three-dimensional structure of Cz in co-complex with the covalent inhibitor **Neq0409** (PDB ID: 4QH6). (B) Cz binding site showing the subsites S1', S1, S2, and S3 (PDB ID: 2OZ2).

The catalytic Cys25 and the central residues involved in binding to the inhibitor are also shown. Water molecules and ligands were removed for clarity. Source: Adapted from AVELAR, L. A. et al. Molecular Design, Synthesis and Trypanocidal Activity of Dipeptidyl Nitriles as Cruzain Inhibitors. **PLOS Neglected Tropical Diseases**, v. 9, n. 7, p. e0003916, 14 jul. 2015; CIANNI, L. et al. Can Cysteine Protease Cross-Class Inhibitors Achieve Selectivity? Can cysteine protease cross-class inhibitors achieve selectivity? *Journal of Medicinal Chemistry*, jul. 2019. <https://doi.org/10.1021/acs.jmedchem.9b00683>.

This attack, depending on the nature of the warhead, can both promote irreversible or reversible binding interaction of such compounds. Usually, reversible inhibition involves non-covalent interaction between the enzyme and the inhibitor, but in the case of reversible cysteine protease inhibitors, inhibition typically includes the reversible formation of a covalent bond between inhibitor and active-site cysteine. Examples of covalent inhibitors are aldehyde peptides, α -diketones, α -ketoesters, α -ketoamides, α -keto acids and nitriles (CIANNI et al., 2019). On the other hand, compounds such as peptidyl diazomethyl ketones, fluoromethyl ketones, epoxides and vinyl sulfones, are capable of binding irreversibly to the catalytic cysteine of Cz by acting as "suicidal" inhibitors (SCHEIDT et al., 1998). K777 was the leader of the first generation of irreversible Cz inhibitors and initially characterized by the Sandler Center for Research in Tropical Parasitic Disease (University of California, San Francisco; SAJID et al., 2011). Besides the ability of this compound to rescue mice of a lethal experimental *T. cruzi* infection and reduce parasite growth in dogs, preclinical safety and toxicology studies revealed substantial side effects when low doses were administered in

primates and dogs (“K777 (Chagas) | DNDi”, 2011; URBINA, 2010). Nowadays, drug repurposing programs of different pharmaceutical companies have recently put forward reversible inhibitors of Cz such as Balicatib (LU et al., 2018), Cz007 (BEAULIEU et al., 2010), Cz008 (BEAULIEU et al., 2010; NDAO et al., 2014; Figure 3).

Figure 3. 2D chemical structures of covalent reversible and irreversible inhibitors of Cz arrived in preclinical phase for CD.

Source: Own author.

This enzyme has a high degree of structural identity with the human enzymes cathepsin K, cathepsin L, cathepsin S and high sequence homology with cathepsin B that are involved in many physiological processes (KRAMER; TURK; TURK, 2017). Therefore, the knowledge generated from Cz inhibition can be transposed to its mammalian counterparts and *vice-versa* (CIANNI et al., 2019). The same concept can be applied to another protozoan parasite CPs recently used as a pharmacological target based on *in vitro* and *in vivo* models, as the cysteine protease B of the *Leishmania mexicana* (LmCPB), the obligate intracellular protozoan parasite that causes the cutaneous form of leishmaniasis (CROFT; COOMBS, 2003; SIQUEIRA-NETO et al., 2018).

The main objective of this Ph.D. thesis is focused on the design, synthesis and biological evaluation of new covalent inhibitors of Cz as trypanocidal agents. In particular, six different key points are taken into consideration:

1. Ponder the influence and importance of S3-P3 interactions for the inhibition of Cz;

2. Understand the role of the cyclopropane moiety in P1 in the bimolecular recognition process between the archetype dipeptidyl nitrile and Cz;
3. Expand the previous series of dipeptidyl nitrile by mapping the S1-S1' subsite of Cz and other CPs;
4. Evaluate the inhibition ability of Cz and the trypanocidal activity of new deconstructed derivatives of Cz008 and Cz009;
5. Estimate sulfonamides as bioisostere of the amides in P2/P3 for the inhibition of Cz and other CPs;
6. Assess the influence of different warheads for a prototype Cz inhibitor compound.

2. CHAPTER I – Leveraging the Cruzain S3 Subsite to Increase Affinity for Reversible Covalent Inhibitors¹

2.1. Background

As discussed above, the inhibition of Cz is an essential strategy for the treatment of CD. In particular, our recent study (AVELAR et al., 2015) showed fundamental interactions of P1, P2 and P3 portions of dipeptidyl nitrile ligand (**Neq0570**) with the respective subsites of the enzyme (S1, S2, S3). Unambiguously, three critical hydrogen bonds were observed between the ligand and the residues Asp161, Gly66 located at S2 and Gln19 located at S1. While P2 and P3 moieties formed mostly hydrophobic interactions with the respective S2 and S3 pockets (WIGGERS et al., 2013).

The S3 region is an open-flat water accessible surface area and therefore usually challenging to exploit the affinity, the S2 pocket has been already used to enhance Cz affinity. However, it has been reported that the residue Ser61, situated at the top of the S3 region, is able to form hydrogen bond interaction with different inhibitors co-crystallized with Cz (for instance, see PDB code 1ME3; (HUANG; BRINEN; ELLMAN, 2003). Accordingly, we performed the design and synthesis of a new series of dipeptidyl nitrile, aimed to evaluate the regioselective interaction with residue Ser61 in the S3 subsite (Figure 4).

¹ This chapter describes joint work with M. Sc. Fabiana Rosini (NEQUIMED/IQSC/USP, University of São Paulo, Brazil) for ITC studies and with Dr. Geraldo Sartori (NEQUIMED/IQSC/USP, University of São Paulo, Brazil) for Molecular Dynamics simulations.

Figure 4. A) Representation of the chemical transformation from **Neq0570** to **Neq0710**.

Possible halogen interaction is represented with a red dashed line. B) The average pose obtained after the molecular dynamic simulation with **Neq0710** covalently bond with cruzain.

PDB code 1ME3; Color for **Neq0710**: Carbon chain is gray; Chlorine is green; Oxygen is red. Protein is represented as a polar surface; from white to red, there is an increment of polarity. The catalytic residue of Cysteine is spotted with yellow color. P1, P2, and P3 represent the substituents of the compound that interact with their respective subsites (S1, S2, and S3) at the enzyme. Source: Figures adapted from: CIANNI, L. et al. Leveraging the cruzain S3 subsite to increase affinity for reversible covalent inhibitors. **Bioorganic Chemistry**, v. 79, p. 285–292, sep. 2018.

2.2. Design, synthesis and biological evaluation

To understand the possibility of halogen and hydrogen bond interaction of meta-substituted phenyl derivatives with Ser61, molecular dynamic (MD) simulations were performed for derivatives of **Neq0570** with different substituents (-OH; -Cl; -CH₃) in *meta*, *ortho* or *para* position of the phenyl ring. Simulations displayed possible hydrogen or halogen bond with the Ser61 when the hydroxyl group or the chlorine atom is in *meta*, as for **Neq0710** and **Neq0717**.

For a better understanding of this hypothesis, we also evaluated the influence of different halogen atoms in *meta*-position of P3 ring. The fluorine derivative molecule (**Neq0802**) and the trifluoromethyl derivative molecule (**Neq0805**) showed high rotational flexibility of ring, similar to that found for **Neq0570**. This phenomenon is an indication of the interaction lost between S3 subsite and P3 ring and thus, between the fluorine atom and Ser61 side chain. On the other hand, for the bromine-derivative compound (**Neq0803**), we observed more sampling for smaller distance, which is

similar to those found for **Neq0710**. Based on these simulations, molecules **Neq0710**, **Neq0717**, **Neq803** and **Neq0804** can probably perform a hydrogen bond or halogen bond interaction with residue Ser61 of the cruzain S3 cavity. Therefore, we synthesized a set of molecular pairs to evaluate *in silico* results by using inhibitor constants (K_i) and thermodynamic profiles (ITC; Figure 5 and Table 1).

Figure 5. Retrosynthetic analysis, general synthesis, structure representations, and Nequimed codes for compound present in chapter I.

Reagents and conditions: (a) HATU, DIPEA, DMF, rt, 18 h; b) formic acid, rt, 18 h; c) HATU, carboxylic acid, DMF, rt, 18 h. Source: Own author.

The synthetic route was developed to optimize the set of substituents to be placed in P1, P2 and P3 that have been defined after the planning and designing studies. All

syntheses were started with enantiopure amino acids. Due to the diversity of building blocks, it was necessary to evaluate different coupling reagents, aiming at the best yield and lower racemization. The peptide coupling reaction was performed twice: first to bind the amino acid (enantiomeric pure, amine-Boc-protected) to the nitrile moiety, and secondly (after removing Boc group) to the corresponding benzoyl acids.

To ensure that no racemization occurred we have synthesized the (R) analogs of **Neq0710**. Enantiomeric purity was analyzed by HPLC equipped with a chiral column (see appendix B).

Kinetic measurements were performed for substances in Scheme 1 to evaluate the affinity of the synthesized compounds. Using pK_i affinity values from Table 1, we performed a Matched Molecular Pair Analysis (MMPA) that provides the opportunity to evaluate molecular modifications for significant structure-activity relationships (SAR; KENNY; SADOWSKI, 2005; TYRCHAN; EVERTSSON, 2017).

The most substantial difference in the observed pK_i values is found in the *m*-H/*m*-I substitution (**Neq570** vs. **Neq0804**) with the benefit of 0.8 log units in favor of the iodine atom in *meta*-position. Moreover, the inclusion of the chlorine atom in the *meta*-position resulted in the second significant difference observed in the series with affinity gain about 0.7 log units (**Neq0570** vs. **Neq0710**). As we expected, the replacement of chlorine, bromine or iodine atom by a trifluoromethyl substituent resulted in a loss of affinity. The inclusion of the nitrogen heteroatom for the formation of the pyridine moiety did not represent the right approach.

In summary, these results point out the direct influence of the Cl, Br and I regiochemistry in the P3 phenyl ring as an adequate strategy to gather a possible halogen bond with the hydroxy group from Ser61. In order to better evaluate this trend, we employed isothermal titration calorimetry to determine thermodynamic parameters of cruzain–dipeptidyl nitrile interactions (Table 1).

As already described in our recent study (PROKOPCZYK et al., 2014), isothermal titration calorimetry (ITC) is a technique used for the thermodynamic characterization of interactions between biomolecules and small molecules, which is vital for understanding the process of molecular recognition. The values of K_d (Table 1) showed the same trend as depicted by pK_i , with substance **Neq0804** having the highest affinity for Cz, followed by substances **Neq0803** and **Neq0710**. The thermodynamic

signatures gathered from ITC data reveal that binding of cruzain inhibitors was driven by favorable enthalpy contributions, with detrimental entropy for all study substances.

Table 1 reports the thermodynamic signatures of the interactions that have, mostly, the same free energy (ΔG) of interaction. The interaction energy relates to the affinity, which is a combination of enthalpy (ΔH) and entropy (ΔS). Classically, enthalpy reflects the strength of the interactions due to the formation of hydrogen bonds and van der Waals, while the binding entropy is a combination of the change in the desolvation and conformational changes after the formation of the bimolecular ligand-cruzain complex (DENG et al., 2011). Results unveil a quasi-perfect omnipresent phenomenon of enthalpy-entropy compensation. Interestingly, the effect of the m-chlorophenyl moiety on binding affinity decreases both binding enthalpy and entropy contributions. To sum up, ITC studies pictured that the mechanism of binding of cruzain inhibitors is driven by favorable enthalpy contributions with a strong phenomenon of enthalpy-entropy compensation.

Table 1- Kinetic parameters (pK_i), pK_d and thermodynamic characterization of cruzain inhibitors.

ID	pK_i^a	pK_d^b (nM)	$\Delta G(\text{kcal mol}^{-1})$	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
Neq0570	6.4 (0.026)	6.6 (0.04)	-9.00	-14.6	5.6
Neq0709	6.6 (0.058)	ND	ND	ND	ND
Neq0710	7.1 (0.020)	6.9 (0.06)	-9.43	-10.7	1.32
Neq0711	6.4 (0.018)	ND	ND	ND	ND
Neq0712	6.6 (0.0099)	6.8 (0.03)	-9.29	-12.8	3.55

ID	pK_i^a	pK_d^b (nM)	ΔG (kcal mol ⁻¹)	ΔH (kcal mol ⁻¹)	$-T\Delta S$ (kcal mol ⁻¹)
Neq0717	7.0 (0.019)	6.8	-9.22	-11.9	2.64
Neq0714	5.9 (0.017)	6.5 (0.11)	-8.83	-12.1	3.30
Neq0802	6.3 (0.023)	6.6 (0.005)	-9.11 (0,005)	-13.9 (0.60)	5.96 (0.59)
Neq0803	6.9 (0.032)	7.0 (0.06)	-9.58 (0,015)	-11.3 (0.35)	1.77 (0.39)
Neq0804	7.2 (0.02)	7.1 (0.03)	-9.76 (0.035)	-12.0 (0.30)	2.28 (0,33)
Neq0805	6.6 (0.02)	6.9 (0.01)	-9.45 (0.015)	-12.7 (0.35)	3.29 (0.38)

Affinity (pK_i) values against Cz. ND – Not Determined. Standard deviations are reported in brackets. Deviations for ΔG are lower than 0.4%; $\Delta H < 4.5\%$; $-T\Delta S < 15\%$. ^a $pK_i = -\log K_i$; Uncertainty = desvpad / ($K_i \cdot \ln(10)$); ^b $pK_d = -\log K_d$; Uncertainty = desvpad / ($K_d \cdot \ln(10)$). Source: Own author.

The closest structural related mammalian cysteine to Cz is cathepsin L (CatL). It is a lysosomal ubiquitous cysteine proteinase (KIRSCHKE et al., 1977) that plays a vital role in degrading endocytosed and intracellular proteins. CatL knockout mice studies provided a series of alterations in the biological processes. (KRAMER; TURK; TURK, 2017) Moreover, CatL upregulation has been reported for many human cancers including ovarian, breast, prostate, lung, gastric, colon, and pancreatic tumours (CHAUHAN; GOLDSTEIN; GOTTESMAN, 1991; SUDHAN; SIEMANN, 2015).

We evaluated the affinity to CatL for the same set of molecules (Figure 6). Results display the same trend found for Cz but with a larger magnitude in ΔpK_i which is due to a stronger halogen bond formation with the backbone peptide carbonyl of Gly66 as already described for a similar class of inhibitors (HARDEGGER et al., 2011).

We could speculate that this carbonyl group is more prone to form a halogen bond for three factors: it can easily create an angle of 180° with the halogen; it has a lower

intrinsic mobility than the hydroxyl group of Gly66 in Cz; it cannot act as hydrogen bond donor leading to be less solvated than a hydroxyl group.

Figure 6. Affinity values and proposed mode of binding for **Neq0803** against CatL.

(A) Affinity (pK_i) values against CatL. $^a pK_i = -\log(K_i)$; Uncertainty = $\text{desvpad}/(K_i \cdot \ln(10))$; (B) The average pose obtained after the molecular dynamic simulation with **Neq0803** covalently bond with CatL (PDB code 2XU1). Color for **Neq0803** and **Gly66**: Carbon chain is gray; Bromine is green; Oxygen is red. Protein is colored in blue the residue Gly66 is represented as balls and sticks (C) ΔpK_i is calculated as the difference between pK_i values; (D) schematic representation of the halogen bond (red dashed line) between Gly66 and the prototype dipeptidyl nitrile bearing a halogen in meta position in P3. Source: Own author.

2.3. Conclusion and perspective

Visual inspection and molecular dynamics simulation have shown that the *m*-chlorophenyl, *m*-bromophenyl, and *m*-hydroxyphenyl moieties have the right orientation inside P3 to interact with the oxygen of Ser61 for Cz or the Gly66 for CatL residue. The results are in good agreement with the MD simulations, once the better compounds are precisely those who present a lesser variation of P3 ring. Indeed, the substitution of a hydrogen atom to an iodine atom in *meta* position in P3 increased the pK_i of 0.8 log unit for Cz and 1.7 for CatL inhibition. Moreover, ITC studies pictured that the mechanism of binding of cruzain inhibitors was driven by favorable enthalpy contributions and with the *quasi*-perfect omnipresent phenomenon of enthalpy–entropy compensation. This finding is of particular interest to guide further SAR for inhibition of cysteine proteases when halogen bonding is implemented in the bimolecular recognition process to increase affinity. Overall, this study underlines the feasibility of achieving higher affinity or selectivity for the desired CP by benefit from structural singularity present in the S3 subsite. Unfortunately, even if we were able to increase the affinity to Cz, none of the compounds described until here exhibit an improvement in trypanocidal activity, respect to the prototype compound **Neq0570**. Nevertheless, results suggest that the design of new cysteine protease inhibitors driven by molecular dynamics is a valuable approach when considering less druggable subsites of the enzyme. Nonetheless, these studies were uniquely crucial in guiding the molecular modifications implemented in the scaffold under investigation, which will be presented in the following chapters.

3. CHAPTER II – Experimental Study and Computational Modelling of Cruzain Cysteine Protease Inhibition by Dipeptidyl Nitriles²

3.1. Background

In the previous chapter (chapter I), we have focused our attention on the P3-S3 interaction for a series of dipeptidyl nitrile with Cz and CatL inhibition. In this chapter, the aim is to understand what the role of the cyclopropane moiety in P1 for the bimolecular recognition process with Cz is. In general, the 1-aminocyclopropanecarbonitrile is a well-known building block used for the synthesis of nitrile CP inhibitors. This moiety is also found in Odanacatib, the only cysteine protease inhibitor to reach clinical phase III (BOGGILD et al., 2015). The cyclopropane moiety confers higher metabolic stability to the 2-aminoacetonitrile group, but its influence on the bimolecular recognition process and in the mode of action (MoA) for the inhibition of Cz was still not fully understood.

3.2. Design, synthesis, computational study, and biochemical evaluation

Structurally similar molecules can present significant differences in potency (or affinity). These discontinuities are known as "activity cliffs" (DIMOVA; BAJORATH, 2016), and it is precisely this phenomenon that we have observed when small changes are applied to the P1 moiety of our prototype dipeptidyl nitrile inhibitor **Neq0570**. Indeed, to better understand the role of the cyclopropane (P1 moiety) in the bimolecular recognition process, we have designed and synthesized two molecular pairs: **Neq0410** and **Neq0409**.

The general synthesis of these two compounds was performed as previously described (Figure 5).

² This chapter describes joint work with M. Sc. Fabiana Rosini (NEQUIMED/IQSC/USP, University of Sao Paulo, Brazil) for ITC studies and with the group of Prof. Jerônimo Lameira (Federal University of Pará, Brazil) for QM/MM calculations.

The modifications implemented in the **Neq0570** inhibitor (Figure 7) negatively modified the binding affinity and the thermodynamic profile of the ligands when co-complexed with cruzain. Substitution of the cyclopropane for geminal dimethyl (**Neq0410**) reduced the affinity over one log unity. Insertion of the cyclopropane in P1 on **Neq409** increased the potency of 0.3 log units. For comparisons between the pairs to be significant, the thermodynamic parameters were evaluated. The pair **Neq0410-Neq0409** presents a thermodynamic signature in which the introduction of the geminal methylene results in a total detrimental contribution towards binding and the three thermodynamic parameters are unfavorable to the spontaneity of the bimolecular interaction process. On the other hand, the elimination of the cyclopropane moiety in **Neq0570** thereby yielding to **Neq409** contributes positively for the entropic contribution that makes the spontaneous process happen even at the expense of the detrimental contribution of its net enthalpy (Figure 7B). The transformation of the geminal dimethyl moiety to the cyclopropane is favorable for the enthalpy and the entropy of the bimolecular interaction between the inhibitor and Cz.

Figure 7. A) SAR representation for compounds **Neq0409**, **Neq0410**, and **Neq0570**. The ΔpK_i was calculated as the difference of pK_i values (Cz) for the corresponding compounds. B) Net thermodynamic profile for cruzain inhibitors: **Neq0409**, **Neq0410** and **Neq0570**. C) Representative structures obtained from the QM/MM free energy simulations for **Neq0409**. Structures named according to the locations along the 2D PMF.

Colours for the atoms in (C): Carbon chain is grey; Hydrogen is white; Nitrogen is blue; Oxygen is red; Sulfur is yellow. Source: Adapted from DOS SANTOS, A. M. et al. Experimental study and computational modelling of cruzain cysteine protease inhibition by dipeptidyl nitriles. **Physical Chemistry Chemical Physical**, v. 20, p. 24317–24328, set. 2018.

Noteworthy, the insertion of the cyclopropane or geminal dimethyl in the P1 of **Neq0410** led to the same difference in affinity for CatL. To better evaluate the influence of difference substituent in P1 Prof. Lameira and its collaborators have performed a QM/MM calculation to estimate the kinetics and thermodynamics of reversible covalent inhibition of CPs. Briefly, simulation of the reaction inhibition of Cz reveals that reaction proceeds via a concerted mechanism, where the proton transfer from His162 to N1 of **Neq0409** co-occurs with a nucleophilic attack from negatively charged Cys25 at C1 of **Neq0409** (Figure 7C). We could postulate that the difference in potency amongst **Neq0409**, **Neq0410** and **Neq0570** derived from two effects: steric effects of the

dimethyl geminal group and ability of the cyclopropane moiety to conjugate with acceptor substituents (*i.e.*, nitrile)(CRUZ-CABEZA; ALLEN, 2011), herein increasing the nucleophilicity of the vicinal carbon atom.

3.3. Conclusion and perspective

Overall, ITC analysis showed that the cruzain inhibition by **Neq0409**, **Neq0410** and **Neq0570** is enthalpy driven with a clear detrimental contribution of the entropy difference to the free binding energy. Furthermore, an important conclusion from the hybrid QM/MM approach and FESs obtained is that the inhibition reaction of cruzain by **Neq0409**, **Neq0410**, and **Neq0570** follows a concerted mechanism.

These results lead us to explore the S1/S1' of Cz and other CPs to enhance the affinity of dipeptidyl nitrile inhibitors (see chapter III).

Also, considering that nitrile inhibitors of CPs in clinical trials are bearing cyclopropane nitrile as a warhead, we hope that these results may provide valuable information for designing new inhibitors of Cz and other relevant cysteine proteases as biological targets.

4. CHAPTER III – Mapping The S1 and S1' Subsites of Cysteine Proteases for The Synthesis of New Dipeptidyl Nitriles as Trypanocidal Agents³

4.1. Background

A variety of studies have been conducted on optimization strategies for the interactions of different classes of inhibitors with the S1, S2 and S3 binding sites of cruzain and related cysteine proteases. (see chapter I)(AVELAR et al., 2015; CIANNI et al., 2019; SCHMITZ et al., 2019). Nonetheless, far less is known about the possible interactions at S1' for dipeptidyl nitrile inhibitors. The high-resolution crystal structure of Cz shows that there is a large open hydrophobic surface characterized by Trp177 in the primed binding site region (MCGRATH et al., 1995). The design of compounds to exploit this cavity would provide the potential for enhanced enzyme-inhibitor interactions. This concept has already been applied to a class of different dipeptidic vinyl sulfone inhibitors (BRINEN et al., 2000). As Figures 8A and 8C exemplarily illustrates, hydrophobic moieties accommodate in the P3 and P2 pockets while forming hydrogen bonds with Gly66. In particular, the substituents in the current series of the vinyl-sulfone inhibitors predominantly sit on top of the shelf formed by residues Ser139, Met142 and Asp158 rather than adopting an orientation for a strong aromatic–aromatic interactions with Trp177. The nitrile inhibitor 33L (Figure 8B and 8D) does not bear an appropriate substituent that would allow for an interaction with the primed binding region of cruzain. As discussed above (see chapter II) small changes, as the insertion of the bulky substituent, in the P1 position could lead to drastic fluctuation in the pK_i values. By applying a knowledge-based design approach, we have explored different amino acids as possible building blocks for the P1 moiety. Based on a template crystal structure of the dipeptidyl nitrile inhibitor 33L bound to Cz (NEQUIMED/IQSC/USP PDB ID: 4QH6),

³ This chapter describes joint work with the group of Prof. Jürgen Bajorath (University of Bonn, B-it, Germany) for modelling studies and with the group of Prof. Michael Gütschow (University of Bonn, Pharmaceutical Institute, Germany) and with M. Sc. student Fernanda dos Reis Rocho (NEQUIMED/IQSC/USP, University of São Paulo, Brazil) for kinetics measurements. Biological activities were performed by Ph.D. student Daiane Tezuka (NEQUIMED/IQSC/USP, University of São Paulo, Brazil).

structural modifications were explored that might increase the affinity towards the S1' specificity pocket.

Besides that, we have tested this same set of compounds against four human cysteine cathepsins, (CatB, CatL, CatK, CatS) important therapeutic targets for human diseases (KRAMER; TURK; TURK, 2017), and against the cysteine protease LmCPB, novel macromolecular target for *Leishmania mexicana* (MOTTRAM; BROOKS; COOMBS, 1998).

Figure 8. A, C) Crystal structures and 2D interactions of vinyl sulfone derivative K777 with the active site of Cz (PDB-ID: 1F2B); residual number corresponding to cruzain sequences; B, D) Crystal structure and 2D interactions of dipeptidyl nitrile 33L with the active site of Cz (PDB-ID 4QH6); residual number corresponding to cruzipain sequences .

Colours for the atoms in A, B: Carbon chain of the ligand is magenta (left picture) or yellow (right picture); Carbon chain of the protein is grey; Nitrogen is blue; Oxygen is red; Sulfur is yellow. Source: Provided by Christian Feldmann (University of Bonn, B-it, Germany). In pictures C, D: Black dashed lines indicate hydrogen bonds. Green solid lines show hydrophobic interactions.

Therefore, having all of this in our mind, we expanded our previous series of Cz inhibitors to map the S1/S1' subsites.

4.2. Design, synthesis and kinetic evaluation of new dipeptidyl nitriles as cysteine protease inhibitors

By having all this in mind we have applied a knowledge-based design approach, by exploring different amino acids as possible building blocks for the P1 moiety (Figure 9). Modelling studies revealed that dipeptidyl nitriles **Neq0937**, **Neq0940**, **Neq0921**, and **Neq0922** with different lipophilic substitution patterns at the P1 position were assumed to accommodate on the surface of S1' through hydrophobic interactions without interfering in the general mode of binding (Figure 9).

Figure 9. Putative orientation of novel P1 moieties in compounds **Neq0937**, **Neq0940**, **Neq0921**, and **Neq0922** with residues of interest (PDB ID: 4QH6) from the pockets S1 and S1' of Cz.

Colours for the atoms: Carbon chain of the ligand is blue (up-left picture), magenta (up-right picture), green (down-left picture), yellow (down-right picture); Carbon chain of the protein is grey; Nitrogen is blue; Oxygen is red; Sulfur is yellow. Source: Provided by Christian Feldmann (University of Bonn, B-it, Germany).

Compound **Neq0533.1** was adopted as an archetype, with the cyclopropane group at P1 position and phenylalanine as well as a pyrazole moiety for advantageous interactions with the non-primed binding region of the target protease. We mainly used different natural and unnatural amino acids for the P1 moiety and maintained the nitrile warhead. Leucine (Leu) and phenylalanine (Phe) were incorporated (for example, **Neq0937** and **Neq0940**) for aliphatic and aromatic interactions (Table 2). 4-pyridylalanine was implemented to leveraging the affinity by polar interaction with

Asp161 (**Neq0953** and **Neq0954**). Thr-O-Bzl, a great building block for peptide inhibitors, was used as chimera for aliphatic and aromatic interactions (**Neq0921**). Also, removal of the benzyl protecting group from Thr-O-Bzl the so produced alcoholic moiety should allow to evaluate whether a hydrogen bond donor is tolerated in the S1/S1' area. Moreover, it was intended to investigate how the stereochemistry in this region will influence the affinity with Cz (**Neq0941**). Furthermore, to estimate possible non-additivity features in the SARs, the Thr-O-Bzl role in the bimolecular recognition process, we exchanged Phe to Leu and 3-Cl-Phe in P2 by keeping the same fragment in P1 (as **Neq0922** and **Neq0877**).

3-(*tert*-Butyl)-1-methyl-1*H*-pyrazole-5-carboxylic acid is a privileged building block applied for the inhibition of Cz and CatL (ASAAD et al., 2009). Thus, we have explored some possible bioisosteres to increase the affinity and the selectivity towards Cz, such as 7-chloroquinoline carboxylic acid, 1*H*-indole-5-carboxylic acid, or 6-aminonicotinic acid. Also considering the possibility to form a hydrogen bond or halogen bond interaction with the Ser61 as reported in the previous study (chapter I).

Table 2- Structures representation, number identification, p*K_i* values for CatB, CatK, CatL, CatS, Cz and LmCPB.

Cmpd.	Structure	p <i>K_i</i> values or remaining activity (%) at 10 μ M ^{b, c}					
		CatB	CatK	CatL	CatS	Cz	LmCPB
Neq0570		73% ^b	6.4	7.4	7.1	6.3	6.6
Neq0865		5.5	6.2	7.3	7.7	6.9	6.2
Neq0543		5.4	6.4	8.6	6.7	7.4	6.7
Neq0533.1		4.8	6.5	8.2	6.8	7.3	7.1

Cmpd.	Structure	CatB	CatK	CatL	CatS	Cz	LmCPB
Neq0945		93% ^b	5.0	7.6	5.6	6.6	6.4
Neq0569		4.5	8.3	7.6	7.4	7.8	7.3
Neq0956		4.4	96% ^b	5.9	5.9	5.1	5.5
Neq0719		4.9	81% ^b	7.2	6.7	6.7	6.9
Neq0775		89% ^b	6.9	6.6	6.9	7.8	7.3
Neq0708		4.7	n.i. ^c	5.7	6.0	6.2	5.7
Neq0777		5.2	6.2	6.3	7.3	6.9	7.0
Neq0955		n.i. ^c	8.0	6.5	7.6	7.1	6.8
Neq0958		4.5	8.7	7.1	7.3	7.7	7.2
Neq0957		4.6	5.5	5.2	5.0	51% ^b	67% ^b
Neq0937		4.5	6.3	8.5	7.3	7.7	7.8

Cmpd.	Structure	CatB	CatK	CatL	CatS	Cz	LmCPB
Neq0866.1		94% ^b	5.6	6.9	5.9	6.3	6.1
Neq0940		5.1	6.7	8.3	6.9	7.5	7.4
Neq0952		90% ^b	6.0	7.0	6.0	6.5	6.4
Neq0953		4.9	6.3	8.3	7.1	7.5	7.5
Neq0954		4.8	6.5	8.3	7.2	7.5	7.2
Neq0921		5.2	5.7	7.0	6.0	6.3	6.5
Neq0938		n.i. ^c	97% ^b	85% ^b	n.i. ^c	75% ^b	72% ^b
Neq0922		5.1	7.8	7.2	7.3	7.9	7.7
Neq0939		n.i. ^c	n.i. ^c	90% ^b	85% ^b	60% ^b	75% ^b

Cmpd.	Structure	CatB	CatK	CatL	CatS	Cz	LmCPB
Neq0877		6.3	6.0	8.1	6.5	7.2	6.8
Neq0941		92% ^b	86% ^b	5.3	5.3	68% ^b	69% ^b
Neq0943		89% ^b	81% ^b	5.6	4.7	5.4	4.8
Neq0942		92% ^b	7.8	7.0	5.0	7.1	6.7
Neq0944		93% ^b	5.1	88% ^b	83% ^b	5.4	54% ^b
Neq0948		5.2	6.1	8.2	6.6	6.9	6.0

^aThe standard deviation is lower than 15% for all reported pK_i . ^b Percentage of remaining activity at 10 μ M. ^c n.i. = no inhibition observed at 10 μ M. Source: Own author.

Accordingly, we synthesized a new series of dipeptidyl nitriles (Scheme 1 and 2). For final compounds bearing a cyclopropane residue/moiety in P1, the synthesis was carried out as already reported before (see chapter I). The peptide coupling reaction was performed twice: first to connect the enantiomerically pure, Boc-protected P2 amino acid with the aminonitrile moiety, and secondly, after removing the Boc group, to introduce the corresponding aroyl acids (Scheme 1).

Scheme 1- General synthetic scheme.

Reagents and conditions: a) HATU, DIPEA, 1-amino-1-cyclopropanecarbonitrile, DMF, rt, 18 h; b) formic acid, rt, 18 h; c) HATU or TBTU, DIPEA, carboxylic acid, DMF, rt, 18 h; d) DDQ, DCM, rt, 18 h. Source: Own author.

Scheme 2- Retrosynthetic scheme and general synthesis.

Compound **Neq0957** was synthesized from compound **Neq0956** by removing the benzyl group. As a result of promiscuity of the nitrile group under hydrogenolysis, we performed the cleavage under mild oxidative conditions (Scheme 1; WRIGHT; YU; SPENCER, 2001).

To insert different groups in P1, we adopted a different synthetic strategy (Scheme 2). In general, the desired dipeptidyl primary amide was synthesized, followed by dehydration to form the desired dipeptidyl nitrile. Due to the diversity of building blocks, it was necessary to evaluate different dehydrating reagents, aiming at the best yield and prevention racemization. The cleavage of the benzyl protecting group was performed by hydrogenolysis before the conversion of the primary amide to the nitrile, due to the instability of the nitrile group in hydrogen atmosphere. While for compound **Neq0948**, considering the lability of the chlorine atom under hydrogenolysis, we first transformed the primary amide to the nitrile and then removed the benzyl group under mild oxidative conditions (WRIGHT; YU; SPENCER, 2001). The absolute geometry of the P1 group did not change, but, owing to CIP priority rules, the configuration at the α -carbon for the Thr-O-Bzl building block changed two times: (i) in the dehydration step to form the nitrile group and (ii) when the catalytic cysteine attacks the carbon atom of the nitrile warhead to form a covalent bond (Figure 10).

Figure 10. Schematic representation of the change in stereochemistry for compounds bearing Thr or Thr-O-Bn group in P1.

Source: Own author.

The pK_i values were determined for parasite cysteine proteases (Cz, LmCPB) and for human cysteine cathepsins (CatB, CatK, CatL, CatS). They are reported in Table 2. Compounds **Neq0570**, **Neq0543**, **Neq0533.1** and **Neq0569** have already been described as competitive inhibitors that bind reversibly to Cz (AVELAR et al., 2015).

Some of the compounds are Cz nanomolar inhibitors and also exhibit good affinity for LmCPB, CatL and CatK. The application of such inhibitors extends as candidates for antiprotozoal agents and as cysteine cathepsins inhibitors of the human host cell in various disease states. One crucial question is the cross-reactivity of inhibitors for CPs, for which an extrathermodynamic relationship can be formulated for a set of derivatives (MONTANARI et al., 2000; NÚÑEZ; VENHORST; KRUSE, 2012). The nature of the ligand-target interaction governed by the thermodynamic parameter of the free energy change (via the estimation of the dissociation constant) results in extrathermodynamic relationships for the set of derivatives. So, we investigated the degree of linear correlation between Cz and the other CPs by plotting the pK_i data against each other. Results indicated an extrathermodynamic relationship between Cz and LmCPB, while this was not observed for all the other CPs (Figure 11). This finding highlights that the modes of inhibition for this series of compounds are similar to Cz and LmCPB, corroborating the fact that all the structural transformations of prototype compounds **Neq0533.1** and **Neq0569** affected the affinity towards the two protozoa CPs with the same magnitude (Figure 11 and 12).

Figure 11. Plot of pK_i (Cz) vs. pK_i (LmCPB) for the series of dipeptidyl nitrile here discussed. A linear trendline fitted points.

Multiple $R^2 = 0.809$; Adjusted $R^2 = 0.800$. F-statistic: 97.2 on 1 and 23 DF; p-value: 1×10^{-9} . Gray hatch: 95% confidence limit; Dashed red line: prediction interval Source: Own author.

One common approach to SAR analysis is to examine ΔpK_i values associated with particular structural transformations, and these can be specified concisely using the square bracket notation previously described (SILVA et al., 2017). For example, the structural change of the cyclopropane in P1 of compound **Neq0533.1** to the corresponding benzyl (**Neq0937**) can be noted as the [**Neq0533.1**→**Neq0937**] (Figure 12).

As already described for Cz (AVELAR et al., 2015), the exchange of benzoyl to 1-methyl-3-*tert*-butyl-pyrazolyl-carbonyl [**Neq0570**→**Neq0533**] led to potency increases of 0.5 log units. Following this path, we inserted a meta-benzoic ester in the P3 position in the attempt to design a prodrug analogue **Neq0717** (see chapter I). The transformation [**Neq0570**→**Neq0955**] unfortunately displayed a slight increment as compared to the change [**Neq0570**→**Neq0775**]. Hence, compound **Neq0533.1** (pK_i of 7.4 for Cz) has been used as a prototype for mapping S1-P1 interaction on different targets (Figure 12).

Figure 12. SAR summary for S1-P1 interactions.

Values are reported as differences in pK_i and color-coded as red (negative), green (positive), grey (no significant difference, $\Delta pK_i < 0.2$). Source: Own author.

The effects on affinity resulting from stereochemical modifications in P1 of the structural prototype **Neq0533.1** are shown in Figure 12. In general, the stereochemistry of P1 moiety strongly influences the affinity towards all the CPs. The chemical

transformations [**Neq0937**→**Neq0866.1**] and [**Neq0940**→**Neq0952**] decrease the pK_i values for Cz and LmCP both by about one log unit. Likewise, the double stereochemical modification from (*R,R*) benzyl-protected threonine to the (*S,S*) diastereomer [**Neq0921**→**Neq0938**] led to a complete all-target affinity loss. Instead, the structural transformation from the cyclopropane unit to CH attached with a benzyl group [**Neq0533.1**→**Neq0937**] resulted in a significant affinity increment for Cz and LmCPB of 0.4 and 0.7 log units, respectively. Replacement of the P1 cyclopropane linker with CH attached to isopropyl [**Neq0533.1**→**Neq0940**], 3-chlorobenzyl [**Neq0533.1**→**Neq0953**], or even 4-pyridyl [**Neq0533.1**→**Neq0954**] led to a small increase or no significant difference in potency against those two protozoa enzymes. Moreover, the insertion of the benzyl protected threonine in P1 [**Neq0533.1**→**Neq0937**] decreased the affinity towards Cz by tenfold and threefold for LmCPB. Remarkably, replacement by the hydroxybutyl residues led to an almost one hundredfold affinity loss for both enzymes. Mostly, the same trend in affinity was observed for the four mammalian CPs, when the structural modifications in P1 were realized starting from the prototype compound **Neq0533.1** as illustrated in Figure 12. Singularly, the introduction of a benzyl-protected threonine [**Neq0533.1**→**Neq0937**] resulted in a pK_i decrease of 0.4 log units towards CatB. It is possible to speculate that the positive effect is due to a hydrophobic interaction with the S1' subsite as already reported for another series of dipeptidyl nitriles (SCHMITZ et al., 2016).

As previously described (AVELAR et al., 2015), the effects on affinity when replacing the P2 phenylalanine (**Neq0533.1**) with leucine (**Neq0569**) on affinity appears to depend on the substructural context, and this relates to nonadditivity in the SAR. Accordingly, we used compound **Neq0569** as a starting scaffold for another SAR considering P1, P2 and P3 for structural modifications as summarized in Figure 13.

Figure 13. SAR summary starting from compound **Neq0569**.

Values are reported as differences in pK_i and color-coded as red (negative), green (positive), grey (no significant difference, $\Delta pK_i < 0.2$).

Substitution in P3 positioned 1-methyl-3-*tert*-butyl-pyrazole ring with 7-chloro-quinoline (**Neq0708**), or 1*H*-indole (**Neq0958**) preserved the high affinity towards Cz and LmCPB, and it is particularly striking given that this substitution leads to decrease of 1.0 and 0.5 in the pK_i value for CatL. Noteworthy, when the 7-chloro-quinoline moiety was retained in P3 and leucine was exchanged with tryptophan in P2 [**Neq0775**→**Neq0708**], a significant loss in affinity and selectivity was observed. Insertion of a basic moiety, *i.e.* 2-amino pyridine [**Neq0569**→**Neq0955**], produced a substantial reduction in potency for Cz (-0.7) and LmCPB (-0.5). It is worth mentioning that inhibitor **Neq0958** showed a high affinity for CatK (pK_i of 8.7) with a significant selectivity of 1.5 log units for CatK and CatL and over 3 log units for CatB.

In P2 position, the transformation of the leucine moiety to phenylalanine or its derivatives resulted in a loss in affinity up to one log unit for Cz. A similar replacement led to a gain in affinity towards CatL and CatB, and is consistent with previously reported data (SCHMITZ et al., 2016).

For compound **Neq0569**, as for compound **Neq0533.1**, the stereochemistry of the substituent in P1 is fundamental for the bimolecular recognition process. Moreover, the transformation [**Neq0569**→**Neq0922**] retained the pK_i in the same range for Cz while increasing of 0.4 log units for LmCPB.

Nonadditivity in SAR is of considerable interest for testing and validation of models for binding affinity of ligands (CALABRÒ et al., 2016; KRAMER; FUCHS; LIEDL, 2015) and this is illustrated for the six cysteine proteases in Figure 14.

Nonadditivity can be quantified by comparing the ΔpK_i value resulting from a pair of substructural transformations with the sum of ΔpK_i values that result from the individual transformations. The Cz ΔpK_i values for [**Neq0533.1**→**Neq0569**] (0.5) and [**Neq0533.1**→**Neq0921**] (-1.0) are shown in Figure 14. A sum up to -0.5. Nevertheless, [**Neq0533.1**→**Neq0922**], which corresponds the simultaneous application of the pair transformations, is associated with a ΔpK_i value increase of +0.6, thus indicating that the effects of this pair of transformations on Cz affinity are superadditive. The same was true for the effects of these two transformations on the other five CPs. Analogous analysis of the results in Figure 14B displays the effects of two transformations to be superadditive for the entire CP targets investigated herein. These results show that molecular recognition in P2 drives P1-S1 and P1-S1' interactions.

Figure 14. Nonadditivity of SAR for inhibition of Cz, CatL, and LmCPB.

Values are reported as differences in pK_i and color-coded as red (negative), green (positive), grey (no significant difference, $\Delta pK_i < 0.2$).

Pairwise plots for the selectivity towards Cz in relation to other human cathepsins are provided in Figure 15. Chiefly, it is not trivial to achieve a significant selectivity for Cz inhibitors ($\Delta pK_i > 1.0$) over mammalian CPs due to their high structural similarity of the active site. Undeniably, CatB has a different mode of binding due to the larger S2 and S3 pockets (SCHMITZ et al., 2016).

Compounds **Neq0775** and **Neq0942** displayed a significant selectivity toward CatL and CatS, respectively. The selectivity for Cz in case of compound **Neq0775** is driven by the S3-P3 interaction, while for the second is driven by S1-P1. Likewise, the hydrophobic interaction in S1 and S1' with P1 of compounds **Neq0937**, **Neq0953**, and **Neq0877** resulted in a good selectivity over CatK. On the other hand, results indicate how, in general, CatL, CatS and CatK inhibitors could be repurposed for the inhibition of protozoa CPs.

Figure 15. Selectivity pairwise plots.

Values are given in pK_i. X-axis represents the difference in pK_i for the same inhibitor for a pair of CPs. Y-axis represents the mean value of pK_i for the same inhibitor for a pair of CPs. Black dashed line highlights no selectivity. The magenta dashed line highlight a significant selectivity. Positive differences correspond to Cz pK_i values that are greater than those for CatB, CatK, CatL or CatS.

4.3. Biological evaluation

All compounds synthesized were evaluated for their trypanocidal activity against the amastigote form of the Tulahuen *T. cruzi* strain, and the results are presented in Figures 16, 17 and 18. Three compounds (**Neq0940**, **Neq0938** and **Neq0877**) were equipotent with benznidazole as trypanocidal agents. In particular, compounds **Neq0940** and **Neq0877** are both low nanomolar Cz inhibitors and one-digit nanomolar for CatL. Compound **Neq0938** had no affinity for any of the six CPs reported herein, which excluded the possibility that its mechanism of action is similar to compound **Neq0940** and **Neq0877**. Physicochemical properties (*i*_logP, MW, TPSA, LogS) play an essential role for molecular discovery activities. As well, for potential trypanocidal agents, which had been designed as protozoan cysteine proteases inhibitors, physicochemical properties can influence their outcome. Therefore, we have included TPSA, calculated logP and LogS in this discussion.

In general, the substitution of the P3 or P2 moieties from the prototype compound (**Neq0533.1**) did not result in any increment in potency, except compound **Neq0956** (Figure 16). Substitution of Phe for (S,R)-Thr-O-Bn led to a modest trypanocidal potency. Modification in P1 strongly modulates the trypanocidal effect. If considering physicochemical properties, we can detect a trend for this small set where the reduction in lipophilicity corresponds to higher potency, as seen for compound **Neq0940** ($EC_{50} = 15.8$; *i*_logP = 2.3).

Figure 16. Schematic representation of physicochemical properties and SARs for trypanocidal activity.

EC₅₀ calculated for amastigote forms of *T. cruzi* (Tuhaluen strain). CC₅₀ calculated for LLCMK2 strain (host cell). TPSA, ilogP, and Ali_LogS have been computed with the swissADME on-line service (DAINA; MICHELIN; ZOETE, 2017). pK_i is referring to Cz inhibition. Source: Own author.

Single modification in P2 or P3 of compound **Neq0533.1** did not produce any beneficial effect on the trypanocidal potency. However, considering both changes [**Neq0533.1**→**Neq0877**], compound (**Neq0877**) exhibits an excellent trypanocidal potency (Figure 17). In this case, the ilogP is the highest of this series and more than one log unit bigger than for compound **Neq0953**. Noteworthy, compound **Neq0938** has no affinity for CPs and displays the same value for TPSA and ilogP as compound **Neq0877**.

Figure 17. Schematic representation for nonadditivity of SARs for trypanocidal activity from **Neq0533.1** to **Neq0877**.

EC_{50} calculated for amastigote forms of *T. cruzi* (Tuhaluen strain). CC_{50} calculated for LLCMK2 strain (host cell). $TPSA$, $ilogP$, and Ali_LogS have been computed with the swissADME on-line service (DAINA; MICHELIN; ZOETE, 2017). pK_i values are referring to Cz inhibition.

Compounds bearing a leucine moiety in P2 displayed a peculiar behavior (Figure 18). Indeed, debenzoylation of the threonine moiety in P1 led to an increase in potency [**Neq0922**→**Neq0943**] and [**Neq939**→**Neq0942**]. The trypanocidal potency for this set of compounds seems to have no direct correlation with Cz affinity. But again, active compounds have an $ilogP$ lower than 3.0, which appears to be favourable for action on *T. cruzi* but without much reflection on Cz action. However, this surprising result should be pursued when synthesizing new trypanocidal agents.

Figure 18. Schematic representation for nonadditivity of SARs from compound **Neq0922** to compound **Neq0944**.

EC₅₀ calculated for amastigote forms of *T. cruzi* (Tulahuen strain). CC₅₀ calculated for LLCMK2 strain (host cell). TPSA, ilogP, and Ali_Logs have been computed with the swissADME on-line service (DAINA; MICHELIN; ZOETE, 2017). pK_i is referring to Cz inhibition.

Potential cytotoxicity of inhibitors was assessed with the LLCMK₂ cell-based assay, and compounds were evaluated over three days using benznidazole as a control. Cytotoxicity at the highest concentration tested, which did not lead to precipitation (250 µM), was low for the majority of test compounds. The most potent inhibitors of the amastigote *T. cruzi* Tulahuen strains (**Neq0877**, **Neq0938** and **Neq0940**) showed the same range of cytotoxicity (SI > 20) when compared to benznidazole. Therefore, these compounds should be considered as new candidates for *in vivo* test against the infective form of amastigote *T. cruzi* Tulahuen strains.

4.4. Conclusion and perspective

A series of 26 new dipeptidyl nitriles were designed, synthesized and tested against the recombinant cruzain (Cz) to map its S1/S1' subsites. Considering the possible interchangeability with another cysteine protease, the same series of dipeptidyl nitriles was also tested in LmCPB of *Leishmania mexicana*. Other potential targets of such inhibitors are human cysteine cathepsins, which are involved in different disease states affecting human beings. Thus, again, the same series of inhibitors have been tested in cathepsins B, L, K and S (CatB, CatL, CatK and CatS, respectively) that are involved, for instance, in cardiovascular diseases, which may be relevant to Chagas heart disease (MARIN-NETO et al., 2007). Our results demonstrate that inhibition of these cysteine proteases can be achieved by appropriate manipulation of the modeling skeleton of dipeptidyl nitriles. In addition, it was possible to identify trypanocidal agents with effects comparable to benznidazole, the drug of choice used for the treatment of Chagas disease. However, it is essential to underline the difficulties in transferring the enzymatic action to the trypanocidal action. Considering our recent works on Cz inhibition, we should investigate from a biological point of view if the mere inhibition of intracellular cruzipain by dipeptidyl nitriles is likely insufficient to cause cell death. In a perspective way, more efforts should be made to clarify the unfeasibility (up to now) to translate inhibition of Cz to trypanocidal activity.

5. CHAPTER IV – Synthesis and Structure-Activity Relationship of Nitrile-Based Cruzain Inhibitors Incorporating a Trifluoroethylamine-Based P2 Amide Replacement⁴

5.1. Background

Up to this point, we have discussed structure-activity relationships of dipeptidyl nitriles as C_z covalent reversible inhibitors. However, the dipeptidyl nitrile scaffold could be cleaved by different proteases due to its structural similarity with endogenous substrates of proteases (LOFFET, 2002). Hence, the design and synthesis of metabolically stable peptide analogs is an essential issue in bioorganic and medicinal chemistry research. Recently, a powerful new strategy was adopted for replacing the peptide bond with a very useful surrogate: the stereogenic trifluoroethylamine function (SANI; VOLONTERIO; ZANDA, 2007; Figure 19). Undeniably, a trifluoroethyl group can replace the carbonyl of an amide and generate a metabolically stable, poorly basic amine that maintains the excellent hydrogen bond of an amide. The main properties featured by the trifluoroethylamino group are:

- 1) very low NH basicity
- 2) CF₃CH(R)NHR' backbone angle close to 120°
- 3) C–CF₃ bond is substantially polar as to the C=O (SANI; VOLONTERIO; ZANDA, 2007).

The NH of the trifluoroethylamine unit is an excellent hydrogen-bond donor (HBD), because of the strong electron-withdrawing effect exerted by the CF₃ group and could always be considered a good mimic of a peptidic NH. In spite of that, the trifluoromethyl group, contrary to the carbonyl oxygen, is a weak hydrogen-bond acceptor (HBA) (DUNITZ; TAYLOR, 1997). The trifluoroethylamine function can be an adequate peptide bond replacement only if the carbonyl group of the original ligand

⁴ This chapter describes joint work with Dr. Juliana Gomes (NEQUIMED/IQSC/USP, University of São Paulo, IQSC, Brazil) and Dr. Daniela de Vita (NEQUIMED/IQSC/USP, University of São Paulo, IQSC, Brazil) for the synthesis of compounds, Biological activities were performed by the group of Prof. Lucio Freitas Junior (Institute of Biomedical Sciences, ICB, University of São Paulo, Brazil).

amide/peptide-bond is not involved in essential hydrogen-bonding interaction with the receptor.

Odanacatib (to date, the only cysteine protease inhibitor to reach clinical phase III) is an excellent example of this replacement, showing sub nanomolar IC₅₀ values against cathepsin K and outstanding selectivity toward other cathepsins. It displays superior pharmacokinetics and efficacy in an appropriate rhesus monkey model (LI et al., 2006). On the other hand, the substitution of the carbonyl group for the trifluoroethyl group produces a new stereogenic center and two possible diastereoisomers from the same dipeptidyl nitrile (Figure 19).

Figure 19. Schematic representation of bioisosteric replacement of the amide bond in P3/P2 position for the trifluoroethylamino group. Example of successful bioisosteric replacement for cruzain inhibitors.

Data retrieved from: BEAULIEU, C. et al. Identification of potent and reversible cruzipain inhibitors for the treatment of Chagas disease. **Bioorganic & Medicinal Chemistry Letters**, v. 20, n. 24, p. 7444–7449, dez. 2010. Source: Own author.

A published series of nitrile-based cruzain inhibitors as active trypanocidal agents (BEAULIEU et al., 2010) that are structurally related to the cathepsin K inhibitor Odanacatib (GAUTHIER et al., 2008) provided the starting point for this study. The compounds in this series typically feature a substituted biphenyl group at P3. The principal objective of the study was to establish structure-activity relationships (SAR) for cruzain inhibition by the deconstruction of the published inhibitor structures. Secondary goals were to explore non-additivity effects in the SAR and to assess the anti-trypanosomal activity of the compounds against an intracellular form of the of *T. cruzi* Y strain.

5.2. Design, synthesis and SAR of nitrile based cruzain inhibitors

In light of that, we designed and synthesized 24 non-dipeptidyl nitrile covalent inhibitors of Cz and two new dipeptidyl nitriles (Table 3).

Table 3. Nequimed codes and structure representation of compounds designed and synthesized. Green area highlights dipeptidyl nitrile scaffold.

Number	Structure	Number	Structure
Neq0631		Neq0594	
Neq0668		Neq0595	
Neq0630		Neq0629	

Number	Structure	Number	Structure
Neq0587		Neq0671	
Neq0633		Neq0670	
Neq0635		Neq0672	
Neq0636		Neq0669	
Neq0642		Neq0641	
Neq0643		Neq0645	

Number	Structure	Number	Structure
Neq0659		Neq0658
Neq0665		Neq0818
Neq0820		Neq0819
Neq0715		Neq0716

Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

The synthesis of **Neq0818** and **Neq0819** were carried out as described in Chapters I and III. Synthesis of non-peptidic nitrile described in the following chapter was carried in two main steps: formation of 2,2,2-trifluorophenyl amino acid intermediates by reductive amination and then amide synthesis by coupling reagents (Scheme 3).

Scheme 3. Retrosynthetic analysis for non-peptidic nitrile described in this chapter.

Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

Methyl esters were produced from the commercial *D*- or *L*-amino acids, using thionyl chloride in dry methanol, following the procedure previously described (BURTOLOSO et al., 2017). The following step consisted in the preparation of the 2,2,2-trifluorophenyl amino acid intermediates, as previously described with slight modifications such as completely absence of water, and use of DME as a solvent for diastereoselective reduction of the imine to the desired amine with $\text{Zn}(\text{BH}_4)_2$ (HUGHES et al., 2007).

Scheme 4. Synthetic scheme of 2,2,2-trifluorophenyl amino acid intermediates.

Reagents and conditions: (a) SOCl_2 , CH_3OH , reflux, 1 h $\rightarrow$ rt, overnight; (b) $\text{ArC}(=\text{O})\text{CF}_3$, K_2CO_3 , CH_3OH , 50 °C, 18 h; (c) 1M $\text{Zn}(\text{BH}_4)_2$ in DME, $\text{ACN}/\text{CH}_3\text{OH}$ (5:1), -40/-45 °C, 3 h; (d) NaBH_4 , THF, rt, 6 h. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

Briefly, the amino esters were reacted with the appropriate 2,2,2-trifluoroacetophenone, in the presence of potassium carbonate and methanol at 50°C for 18 h, to afford the respective imines. The presence of water, resulting from imine formation, in the basic reaction medium at 50 °C allows the hydrolysis of the methyl ester group, providing the potassium salts of the 2,2,2-trifluorophenyl imines. Due to their instability, all imines were used in the next step (diastereoselective reduction) without purification.

The reduction with NaBH_4 afforded, for the *L*-amino acid derivatives, the R,S diastereoisomers (*anti* isomers), reflecting the fact that: the *re* face is sterically more hindered than the *si* face for the more stable conformation of these salts (HUGHES et al., 2007). This is due to the presence of the R_1 group of the *L*-amino acid moiety (phenyl, aryl, isopropyl), allowing the attack of the hydride ion to be, preferably, on the *si* side of the imine (HUGHES et al., 2007). On the contrary, to obtain the S,S diastereoisomers (*syn* isomers) the imines were reduced with $\text{Zn}(\text{BH}_4)_2$. In that case, the formation of the *syn* isomer as a major diastereoisomer can be explained by complexation of Zn^{2+} with the nitrogen and oxygen atoms at the 1,3-positions of the imine, respectively, making the *si* face sterically more hindered. When the starting material is the *D*-amino acid, the reduction of the corresponding imine gives the S,R diastereoisomer (*anti* isomer) with NaBH_4 and the R,R diastereoisomer (*syn* isomer) with $\text{Zn}(\text{BH}_4)_2$.

In general, we observed that NaBH_4 reduction of the imine produced the desired amines with a diastereomeric excess over 95%, while when using $\text{Zn}(\text{BH}_4)_2$ in THF the diastereomeric excess was lower than 80%. Optimization of the reaction lead us to discover DME, $\text{ACN}/\text{CH}_3\text{OH}$ (5:1) as the best solution to increase the diastereomeric excess up to 90%. The diastereomeric excesses in favor of the *syn* or *anti* isomer were measured by HPLC of the final compounds. If necessary, final products were purified by HPLC, equipped with a chiral column, to afford the desired outcome with a *d.e.* > 99% and *e.e.* > 99%. To confirm the absence of epimerization in the chiral center of the amino acids during the synthetic steps, phenylalanine was employed as a model in this study. Therefore, racemic phenylalanine was used as a starting material, and the final compounds, after reduction (with NaBH_4 or $\text{Zn}(\text{BH}_4)_2$) and amine coupling, were analyzed by HPLC which confirmed the integrity of the stereogenic center with an enantiomeric excess >99% (see appendix).

After imine reduction, the amino acids were coupled with the appropriate amine, using HATU as a coupling agent to afford the desired product in good yield (Scheme 5).

Scheme 5. Synthetic scheme for the coupling reaction.

Reagents and conditions: (a) HATU, DIPEA, rt 22, h. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

Finally, compounds **Neq0665** and **Neq0820** were obtained by a Suzuki cross-coupling reaction of the aryl bromide intermediates (Scheme 6). According to the procedure (BANDINI et al., 2009), the bromides reacted in the presence of sodium carbonate with the suitable boronic acid pinacol ester, using PdCl_2dppf as a catalyst in DMF, at 80°C for 3 h.

Scheme 6. Synthetic scheme for Suzuki cross-coupling reaction.

Reagents and conditions: (a) suitable boronic acid pinacol ester, PdCl_2dppf , DMF, 80°C , 3 h, argon atmosphere. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

Measured pK_i values for inhibition of Cz and CatL are given in Table 4. The most potent compound (**Neq0659**; $\text{pK}_i = 9.2$) synthesized in the course of the current study is the

des-methylsulfonyl analog of a cruzain inhibitor for which a pIC_{50} value of 9.7 has been reported (CHENG CHEN et al., [s.d.]).

Table 4. Enzyme inhibition.

Nequimed Code	pK_i (Cz) ^a	pK_i (CatL) ^a
Neq0570	6.6	7.4
Neq0587	6.9	5.9
Neq0594	5.6	ND
Neq0595	5.1	ND
Neq0629	5.0	5.0
Neq0630	6.4	6.2
Neq0631	6.0	6.8
Neq0633	5.6	6.2
Neq0635	7.4	ND
Neq0636	5.7	6.6
Neq0641	7.2	6.2
Neq0642	8.1	6.1

Nequimed Code	pK_i (Cz) ^a	pK_i (CatL) ^a
Neq0643	8.8	8.3
Neq0645	< 5.0	ND
Neq0658	6.6	ND
Neq0659	9.2	5.8
Neq0665	7.1	6.0
Neq0668	5.4	ND
Neq0669	5.5	ND
Neq0670	6.4	6.6
Neq0671	5.4	5.8
Neq0672	6.7	ND
Neq0715	< 5.0	5.9
Neq0716	< 5.0	6
Neq0818	7.3	ND
Neq0819	7.3	ND
Neq0820	8.8	7.6

For all compounds, the standard error of the K_i is lower than 10%. ^a $pK_i = -\log_{10}(K_i)$; ND = not determinate. Source: Own author.

The principal objective of this study was to perform a SAR around known trifluoroethylamine-based cruzain inhibitors to achieve structures of lower molecular complexity (HANN; LEACH; HARPER, 2001) with high affinity for Cz and good antiparasitic activity.

What's more, we used different SAR representations in this study, and Table 5 provides the key for the analyses.

Table 5. Key for structural transformations depicted in Figures 20.

Label	Transformation	ΔN_{nH} ^a	Representation in Figure 20	Representation in Figure 21
A	[P1-cyclopropane→P1-benzyl(S)]	5	————
B	[P3-phenyl→P3-4-biphenyl]	6	-----
C	[P3-S→P3-R]	0	N/A ^b
D	[P2-Leu→P2-Phe]	3	————	N/A ^b

^a Change in the number of non-hydrogen atoms. ^b Not applicable. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

The basis of the lead-likeness concept is that optimization of leads typically results in increased lipophilicity and molecular size. These parameters may be regarded as the primary physicochemical risk factors in drug design (KENNY, 2019; TEAGUE et al., 1999). The structural modifications for which the most substantial increases in affinity

are accompanied by the smallest increases in risk can be regarded being the most efficient in the context of hit or lead optimization (KENNY, 2019). This is the basis of group efficiency (GE; SAXTY et al., 2007). A highly efficient structural transformation can also be seen as a type of activity cliff (MAGGIORA, 2006; STUMPFE; BAJORATH, 2012). One limitation of GE as a tool for SAR analysis is that it is not defined for structural transformations such as inversion of configuration, aza-substitution and amide reversal for which there is no net change in the number of non-hydrogen atoms. The published cruzain inhibitors (BEAULIEU et al., 2010) were deconstructed to the structural prototype **Neq0635** by truncation of P3 biphenyl substituents to phenyl and by replacement of the benzyl substituted P1 linker with cyclopropane moiety. Compound **Neq0672**, in which the P3 chiral center of **Neq0635** is *S* rather than *R*, and the dipeptide nitrile **Neq0818** were also used as structural references. The effects of synthetic elaboration are summarized by plotting pK_i versus the change in number (ΔN_{nH}) of non-hydrogen atoms relative to the reference structure (Table 5). Plotting against ΔN_{nH} rather than N_{nH} allows the dipeptide nitrile SAR to be visualized in the same frame of reference as the other inhibitors. The gradient of the line linking two points quantifies the sensitivity of affinity to an increase in molecular size (KENNY, 2019). For a structural transformation associated with a net change in molecular size, the extent to which the different lines, represent the structural transformation, are parallel gives a visual indication of the degree to which SAR is additive (Figure 20).

Figure 20. The sensitivity of potency (pK_i) to structural modifications A, B, and D (see Table 5 for key).

Numbers in bold refer to Nequimed numbers. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

The [phenyl→4-biphenyl] transformation at P3 (solid black line) leads to increases in pK_i for trifluoroethylamines regardless of the P3 stereochemistry. In contrast, the [cyclopropane→S-benzylmethylene] transformation at P1 leads to a rise in pK_i when the P3 configuration is S but a decrease in pK_i when this is R. The [cyclopropane→S-benzylmethylene] transformation at P1 does not affect the affinity of the dipeptide nitrile **Neq0818**. Exchange of the P2 leucine (solid red line) for phenylalanine leads to decreases in affinity ranging from 0.7 to 1.4 for the three reference compounds; as already reported above for dipeptidyl nitrile (see chapter III).

Substitution of the P3 substituents with a halogen typically leads to small decreases in affinity, in contrast to the positive trend displayed in chapter I for dipeptidyl nitrile. The pK_i values for **Neq0665**, **Neq0636** and **Neq0669** are all within 0.3 log units of the respective values for the compounds from which they were derived. Aza-substitution

of the P3-biphenyl is well tolerated, and **Neq0820** is only 0.4 log units less potent than **Neq0659**. Despite no significant change, a pyrimidine is a fascinating group in the medicinal chemistry field. The pyrimidine increased the solubility of the molecule, without modifying the primary interaction with the hydrophobic pockets of the protein. Replacement of the P2 phenylalanine by O-methyltyrosine for **Neq0594** and **Neq0631** resulted in decreases in pK_i of 0.5 and 0.6 respectively. In contrast, the [**Neq0633**→**Neq0630**] transformation leads to an increase in pK_i of 0.8 log units, which illustrates how the P1-substituent can influence the P2-substituent SAR. Compound **Neq0671** was observed to be at least 0.4 log units more potent than **Neq0629** and this preference for the S configuration at P1 is consistent with what has been reported for analogs substituted with 2-fluoro-4-cyanobenzyl at P1.

The cruzain inhibition SAR associated with the structural transformations A, B and C (defined in Table 5) is illustrated in Figure 21. Opposite faces of the cube in Figure 21 represent the application of the two structural transformations to starting points that are connected by the third structural transformation. The difference between ΔpK_i values associated with parallel sides of each cube face quantifies the extent to which the associated SAR is non-additive while the dependence of the ΔpK_i values on the order in which structural transformations are applied indicates whether the SAR is subadditive or superadditive. This can be illustrated by the effects of using structural transforms A and C to **Neq0672**, which are depicted in the front face of the cube in Figure 21. The difference (1.7 log units) between the ΔpK_i values corresponding to each structural transformation shows that the associated SAR is strongly non-additive.

Figure 21. Nonadditivity in SAR for structural transformations A, B, and C (see Table 5 for key).

Numbers in bold refer to Nequimed numbers. Number in brackets refer to pK_i values.

The results shown in Figure 21 are presented in Table 6 with the corresponding values of δ which quantify the non-additivity for each pair of transformations. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

Table 6. Nonadditivity in SAR for structural transformations A, B, and C (see Table 5 for key).

Initial structure	Transformations ^a		$\Delta pK_i[\text{I}]$ ^b	$\Delta pK_i[\text{II}]$ ^c	$\Delta pK_i[\text{I and II}]$ ^d	δ ^e
	I	II				
Neq0672	A	B	-0.3	+0.5	-0.1	-0.3
Neq0635	A	B	+1.4	+0.7	+1.8	-0.3
Neq0672	A	C	-0.3	+0.7	+2.1	+1.7
Neq0641	A	C	-0.6	+0.9	+2.0	+1.7
Neq0672	B	C	+0.5	+0.9	+1.4	0
Neq0645	B	C	+0.2	+2.4	+2.8	+0.2

^a Defined in Table 5; ^b ΔpK_i referring to the transformation I; ^c ΔpK_i referring to transformation II; ^d ΔpK_i referring to the change I and II applied to initial structure; ^e Degree of nonadditivity, if

$\delta < 0$ subadditive, if $\delta > 0$ superadditive. Source: Adapted from GOMES, J. et al. Synthesis and structure-activity relationship of nitrile-based cruzain inhibitors incorporating a trifluoroethylamine-based P2 amide replacement. **Bioorganic & Medicinal Chemistry**, v. 27, n. 22, p. 115083, nov. 2019.

One interpretation of the superadditive SAR observed for the A and C transformations is that having the P3 substituent with the *S* configuration enables the P1 benzyl substituent to interact more effectively with the protein than for the *R* configuration. Nevertheless, caution is needed when interpreting nonadditivity in terms of molecular interactions, because the phenomenon is inherently non-local in nature.

Compound **Neq0659** shows significant selectivity for Cz over cathepsin L, and the pK_i values against these cysteine proteases differ by 3.4 log units. This is consistent with what has been reported for structural analogs of this compound (BEAULIEU et al., 2010). The corresponding pK_i differences (Cz - CatL) for compounds **Neq0642** (2.0) and **Neq0643** (0.5) show that both P3 biphenyl and P1 benzyl influence selectivity. The P3 configuration also influences selectivity as exemplified by the pK_i differences (Cz - CatL) for **Neq0641** (1.0) and **Neq0645** (-0.2). Aza-substitution of the P3 biphenyl of **Neq0659** leads to reduced selectivity as shown by the pK_i values of 8.8 (cruzain) and 7.6 (CatL) measured for **Neq0820**.

Unfortunately, meaningful SAR cannot be derived for the cell-based assay results, because it was only possible to measure EC_{50} values for the two compounds **Neq0715** and **Neq0716**, (11 μ M and 26 μ M respectively) evaluated in this study. In general, failure of enzyme inhibitory activity to translate to activity in a cell-based assay should be anticipated, especially for an intracellular parasite, and may reflect poor permeability or an enzyme inhibition model that is not relevant in the cellular context (AVELAR et al., 2015). Although neither compound shows significant cruzain inhibition ($pK_i < 5$), the EC_{50} values for **Neq0715** (11 μ M) and **Neq0716** (26 μ M) are too high to allow inferences to be drawn as to whether or not their effects in the cell-based assay are due to cruzain inhibition. As for the class of inhibitors described above, it is not possible to predict trypanocidal activity by Cz inhibition ability.

5.3. Conclusion and perspective

The structure-activity relationship for a series of cruzain inhibitors reveals a significant degree of superadditivity, which can be visualized by plotting pK_i against several non-hydrogen atoms. Moreover, we show that plotting affinity against molecular size provides a means to visualize both the molecular size efficiency of structural transformations and the nonadditivity. Analysis of the structure-activity relationship shows that the biphenyl P3-substituent that is present in the fundamental starting point for the study can be truncated to phenyl with the loss of only 0.4 log units of affinity. It is also shown how the stereochemistry of the trifluoromethyl group in P2/P3 strongly influences the affinity to Cz and the selectivity toward CatL. When comparing with the dipeptidyl nitrile reported above (chapter I, II and III) the bioisostere replacement of the amide bond with the trifluoromethylamine group and (?) lead to one-digit nanomolar inhibitors of Cz with over one-thousand-fold selectivity for CatL. However, it is important to stress that for this class of compound, as for dipeptidyl nitrile (see chapter III), no direct translation from Cz inhibition to anti-trypansomal activity was observed.

6. CHAPTER V – N-Sulfonyl Dipeptide Nitriles as Inhibitors of Human Cathepsin S: In silico Design, Synthesis and Biochemical Characterization⁵

6.1. Background

As mentioned before (chapter **IV**), over the last year our group focused on the synthesis of new derivatives where the carbonyl group in P3/P2 position is replaced with a trifluoromethyl group ($-\text{CF}_3$) (BURTOLOSO et al., 2017) and where the affinity is maintained or increased. However, this kind of replacement brought two challenges: increase number of chiral centers and higher cost of production. Therefore, we focused our attention on the synthesis of new inhibitors by reducing the number of stereogenic centers and synthetic feasibility of sulfonamides. Sulfonamide motif appears in 111 approved drugs or agents in clinical trials. The total number of organic molecules containing this functional group is enormous (SEHGELMEBLE et al., 2012). Besides that, sulfonamides are not conventional bioisostere of amide due to the presence of two oxygen bind to the Sulfur atom and due to the loss of planarity. Sulfonamides are known to have conformational preferences in which the nitrogen lone pair bisects the $\text{O}=\text{S}=\text{O}$ angle in the Newman projection (SEHGELMEBLE et al., 2012). Moreover, it has been already reported that the structural replacement of the peptide bond for a sulfonamide maintained the affinity toward Cz (Figure 22; SCHEIDT et al., 1998).

⁵ This chapter describes joint work with the group of Prof. Jürgen Bajorath (University of Bonn, B-it, Germany) for modelling studies and with the group of Prof. Michael Gütschow (University of Bonn, Pharmaceutical Institute, Germany) for kinetics measurements on human cathepsins and for the synthesis of **Neq0934**, **Neq0935**, **Neq0936** and **Neq0937**.

Figure 22. (a) Newman project of a sulphonamide bond; (b) bioisosteric replacement of the amide bond to sulfonamide bond in P3/P2 region. Example of amide bond replacement from **K777** to **K777.S**; (c) Torsional profile of **Neq0830** performed with “Molecular Operating Environment” (MOE), version 2018.0101.

Source of (a) and (b): Own author. Source of (c): Calculated by Erik Gilberg (University of Bonn, b-it, Germany).

6.2. Design, synthesis and biological evaluation of *N*-Sulfonyl dipeptide nitriles

For a better assessment of the effect of the sulphonamide bond, we designed and synthesized 15 new *N*-sulfonyl dipeptide nitriles. Assuming that *N*-sulfonyl dipeptide nitriles will have the same mode of binding on Cz as on dipeptidyl nitriles, we used our previous results to choose different moieties in P1, P2 and P3 (see chapter III).

Cyclopropane or benzyl moieties have been selected for P1 position, while L-Leu or L-Phe have been inserted in P2. As a result of the loss of planarity when the amide bond is replaced with a sulfonamide bond, 8 distinct modifications have been used in P3 to evaluate their contributions to the affinity of N-sulfonyl dipeptide nitrile for Cz (Figure 23). The synthesis of *N*-sulfonyl dipeptide nitriles is forthright. As for the synthesis of dipeptidyl nitriles (see chapter I and III) it consists of two main steps: the free amine nitrile building block was coupled with the Boc-amino acid; after removal of the Boc protecting group, the free amine reacted with the corresponding sulfonyl chloride (Figure 23).

Figure 23. Retrosynthetic scheme, synthetic scheme, structure representation and Nequimed codes *N*-sulfonyl dipeptide nitriles.

Source: Own author.

In general, pK_i values display an almost flat SAR for the *N*-sulfonyl dipeptide nitriles against Cz, where 12 compounds out of 15 have a pK_i of about 6.5 ($\Delta = \pm 0.4$; Table 7).

Table 7. Nequimed code, substitution definitions, and pK_i values.

Compound	R ¹	R ²	R ³	R ⁴	pK_i^a (Cz)
Neq0781	<i>i</i> -Pr	H	H	H	6.5
Neq0782	<i>i</i> -Pr	H	Cl	H	6.5
Neq0831	<i>i</i> -Pr	H	Ph	H	7.0
Neq0832	<i>i</i> -Pr	H	4-F-Ph	H	6.9
Neq0827	<i>i</i> -Pr	H	H	Ph	5.4
Neq0780	<i>i</i> -Pr	H	Cl	H	6.4
Neq0830	<i>i</i> -Pr	H	Ph	H	6.7
Neq0848	<i>i</i> -Pr	H	Br	H	6.6
Neq0838	<i>i</i> -Pr	H	H	Ph	6.8
Neq0829	<i>i</i> -Pr	H	H	4-F-Ph	6.5
Neq0936	<i>i</i> -Pr	Cl	H	H	5.9
Neq0779	Ph	H	Cl	H	6.1
Neq0934	Ph	H	Ph	H	6.4
Neq0935	Ph	H	H	Ph	6.1
Neq0933	Ph	Cl	H	H	6.5

Reagents and conditions. a) trifluoroacetic anhydride, CH₂Cl₂; b) i) formic acid, rt, 20 h, (ii) corresponding (L)-amino acid, HATU, DIPEA, DMF, rt, 22 h; b) formic acid, rt, 18 h; c) (i) formic acid, rt, 20 h, (ii) corresponding sulfonyl chloride, DIPEA, CH₂Cl₂, 0 °C to rt, 20 h. ^a $pK_i = -\log_{10}(K_i)$. The standard error is lower than 10% for all the measurement. Source: Own author.

We can hypothesize that this effect is due to the torsional profile of the sulfonamide bond. It probably induces a different mode of binding respect to dipeptidyl nitrile inhibitors. Indeed, replacement of the amide bond or trifluoromethylamine moiety for a sulphonamide is detrimental for the affinity as depicted in Figure 24. On the other hand, insertion of the meta biphenyl moiety in P3 exhibits an increase of 0.3 log units [**Neq0934** → **Neq0830**] or 1.6 log units [**Neq0827** → **Neq0831**] in affinity (Figure 24). Overall, we found an interesting positive nonadditivity SAR for the double transformation [**Neq0934** → **Neq0830**] which evidences how the S1 group can shape

the general mode of binding of the P3 meta biphenyl group. In addition, none of the new *N*-sulfonyl dipeptide nitriles showed any significant trypanocidal activity when tested against the amastigote form of *T. cruzi*.

Figure 24. Schematic representation of bioisosteric replacement of the P2/P3 amide and its effect on the affinity for Cz.

ΔpK_i was calculated as the difference of pK_i values. Source: Own author.

The same set of compounds was tested against four human cathepsins (CatB, CatK, CatL and CatS). Results show that most of the *N*-sulfonyl dipeptide nitriles are nanomolar inhibitors to CatS. They display a good selectivity as well toward CatK, CatL and CatB (Table 8).

Table 8. Inhibition of human cathepsins by N-sulfonyl dipeptide nitriles.

pK_i (μM) ¹ or residual activity (%) ²				
Compound	CatB	CatK	CatL	CatS
Neq0781	84%	7.2	6.3	7.2
Neq0782	4.6	7.5	6.9	7.4
Neq0831	4.3	7.4	6.4	7.1
Neq0832	4.3	6.9	6.0	6.9
Neq0827	92%	6.2	6.3	5.9
Neq0780	3.8	7.3	6.6	7.5
Neq0830	87%	7.6	6.6	8.4
Neq0848	83%	7.1	6.8	7.1
Neq0838	86%	7.6	6.6	7.0
Neq0829	97%	5.7	99%	59%
Neq0936	82%	6.7	6.6	6.8
Neq0779	4.5	6.0	7.0	7.6
Neq0934	86%	5.9	7.0	7.6
Neq0935	5.1	6.3	6.8	7.1
Neq0933	4.7	5.6	6.8	6.7

¹ $pK_i = -\log_{10}(K_i)$. The standard error is lower than 10% for all the measurements. ²The remaining activity at 10 μM inhibitor concentration obtained in duplicate measurements is noted. Source: Own author.

Through modelling studying, we have evaluated how these compounds could interact with S1, S2 and S3 of CatS. We proposed a mode of binding of the *N*-sulfonyl dipeptide nitrile, **Neq0831**, where P2 group is flipping to the S3 subsite and the P3 is interacting

with the P2 moiety as seen as for crystallized sulfonyl nitrile inhibitor in the active site of cathepsin S (PDB-ID: 2FQ9; Figure 25). In this proposed conformation the oxygen atoms of the sulfonamide bond could form hydrogen bonds with aliphatic polarized CH-groups of Gly133, Asn158 and Gly160. Too, the biphenyl moiety in P3 could form edge-to-face and π -stacking interactions with Phe67 and Phe205, while the dihedral angle of the sulfonamide bond is in an ideal conformation (~ 55 - 65° ; Figure 25 and Figure 26). As for Cz inhibition, **Neq0831** is the most potent inhibitor against CatS ($K_i < 10.0$ nM), while *N*-sulfonyl dipeptide nitriles bearing Phe in P2 illustrate the highest selectivity against CatK. This is probably due to the bulky effect generated by the Phe in P2 (Figure 27).

Figure 25. Proposed binding mode of *N*-sulfonyl dipeptide nitrile Neq0830 in the active site of CatS.

Proposed binding mode of *N*-sulfonyl dipeptide nitrile **Neq0830** (green) superposed with a crystallized sulfonyl nitrile inhibitor (cyan) in the active site of cathepsin S (PDB-ID: 2FQ9). The protein surface is depicted in transparent grey. Ligand- and residue atoms are shown in stick representation, and specificity pockets are numbered according to the Schechter-Berger nomenclature. Source: Created by Erik Gilberg (University of Bonn, b-it, Germany).

Figure 26. Proposed interactions of the *N*-sulfonyl moiety in the active site cleft of cathepsin S (PDB-ID: 2FQ9).

The oxygens of the sulfonyl substructure are forming a hydrogen bond network with polarized carbon atoms of residues Gly133, Asn158 and Gly160 of the S2 pocket with a preferred torsion angle of the sulfonamide. Source: Created by Erik Gilberg (University of Bonn, b-it, Germany).

Figure 27. Comparison of binding modes of compound **Neq0831** (green) in (a) cathepsin S (PDB-ID: 2FQ9) and (b) cathepsin K (PDB-ID: 4X6H).

Crystallized ligands are shown in cyan. The surface representation of the proteins is colored according to their lipophilicity (lipophilic: red, hydrophilic: blue). Specificity pockets are numbered according to the Schechter-Berger nomenclature. Source: Created by Erik Gilberg (University of Bonn, b-it, Germany).

6.3. Conclusion and perspective

We have designed and synthesized 15 new non-dipeptidyl nitriles, bearing a sulphonamide bond in P2-P3 position to evaluate the sulfonamide amide as possible bioisostere for Cz inhibition. Most of the new *N*-sulfonyl dipeptide nitriles displayed an excellent affinity for Cz. This underlines the possibility of replacing the amide for a sulphonamide bond. However, the comparison of this set of inhibitors with others (presented in chapters III and IV) results in a general loose of affinity and a flat SAR. These phenomena can arise from a different mode of binding of the *N*-sulfonyl dipeptide nitriles. Indeed, the optimal bond angle of the sulphonamide is around 60 ° while for amide or trifluoroethylamine bond is about 110–120 °. At the same time, the same set of compounds displayed a great ability to inhibit CatS with excellent selectivity over the other human cathepsins. Modelling studies and visual inspection of different crystallographic structures of CatS co-crystallized with nitrile inhibitors suggested a different mode of binding.

Overall, this work creates the base for a new class of nitrile Cz inhibitors. In future it needs to be more exploited to enhance the affinity toward protozoan CPs and to demonstrate the hypothetical mode of binding here proposed. Considering the synthetic accessibility of *N*-sulfonyl dipeptide nitriles, we conclude that a more extensive library of compounds should be created to supplant the use of highly not metabolic stable dipeptidyl nitriles as Cz inhibitors.

7. CHAPTER VI – A comparative study of warheads for the design of cysteine protease inhibitors⁶

7.1. Background

In general, cysteine protease inhibitors can be divided into two categories: covalent and non-covalent inhibitors. Also, covalent inhibitors can be sub-divided into reversible or irreversible inhibitors. As already described (SCHNEIDER et al., 2015), the difference between reversible and irreversible inhibitors can be expressed as the free reaction energy (Figure 27a). Indeed, for a strongly exergonic reaction, the half-time of the covalently bound adduct (E-I) tends to be infinite (irreversible warhead): By contrast, for a weakly exergonic reaction, the half-time is longer than for a non-covalent interaction, but the reaction remains reversible (Figure 28a).

The nitrile group is considered to be a particularly useful warhead for cysteine protease inhibition on account of its metabolic stability and polarity, and a small contribution to molecular size (DEATON; KUMAR, 2004). For those reasons, our research group focused mainly on inhibitors of Cz bearing nitrile as a warhead. Concurrently, even if the reversibility of binding may reduce the risk of adverse effects, irreversible binding can result in prolonged duration of action (GEHRINGER; LAUFER, 2019), as for vinyl sulfone derivatives (K777). Figure 28b shows the most employed warheads for cysteine protease inhibition. Not surprisingly, nitrile is the most widely used warhead in crystallographic structures, followed by vinyl sulfone, halomethyl ketone and epoxysuccinate (CIANNI et al., 2019).

⁶ This chapter describes joint work with Dr. Jean F. R. Ribeiro (NEQUIMED/IQSC/USP, University of São Paulo, Brazil) for kinetic characterization. Compounds were synthesized by Dr. Daniel G. Silva (NEQUIMED/IQSC/USP, University of São Paulo, Brazil).

Figure 28. A) energy diagrams of inhibition mechanisms for covalent inhibitors: black lines for the reversible mechanism, red lines for irreversible mechanism; B) list of the most common reversible (blue) and irreversible (red) warheads used for CPs inhibition.

Source: Figure adapted from CIANNI, L. et al. Can Cysteine Protease Cross-Class Inhibitors Achieve Selectivity? Can cysteine protease cross-class inhibitors achieve selectivity? **Journal of Medicinal Chemistry**, jul. 2019. <https://doi.org/10.1021/acs.jmedchem.9b00683>.

In this study, we wanted to explore how different warheads affect the affinity to Cz and their trypanocidal potency. Therefore, we first investigated the transferability of the published effects of warhead replacement. Secondly, we evaluated warheads such as the oxime that are based on a carbon-nitrogen double bond. Thirdly, we explored the potential of substitution for mitigating potential liabilities (e.g. instability or irreversible binding) associated with warheads.

7.2. Design, synthesis and biological evaluation for warhead replacement

First, we replaced the benzoic acid group in P3 (**Neq0409**) to form the equivalent N-Cbz-protected (**Neq0500**) to facilitate the synthesis of compounds bearing different warheads (Figures 29A, 29B). This transformation did not result in any loss of affinity to Cz. Therefore, the nitrile **Neq0500** ($pK_i = 6.3$) was used as the reference compound for this study. On the contrary of what described above for the pair **Neq0570** – **Neq0409** (see chapter II), the cyclopropane analog of **Neq0500**, so-called **Neq0655** ($pK_i = 5.2$), is an order of magnitude less potent (Figure 29).

Figure 29. A) Schematic representation of the structure modification from **Neq0410** to **Neq0500**; B) Schematic representation of the SAR considering molecular pairs between **Neq0500** and the corresponding compound bearing a different warhead. Color highlights the difference in pK_i for Cz; red area highlights a decrease in affinity; green area highlights an increase in affinity. D) Table reports pK_i for Cz, pEC_{50} , pCC_{50} and k_{on} and k_{off} in case of slow binding inhibition.

^a Reversibility assayed; ^b Irreversibility assayed. Source: Own author.

The aldehyde **Neq0640** ($pK_i = 8.1$) is almost two orders of magnitude more potent than **Neq0500**, while replacement of the P1 methylene linked with cyclopropane (**Neq0656**) resulted in a reduction in affinity by nearly one log unit respect to **Neq0500**.

The differing effects of the [nitrile→aldehyde] replacement for **Neq0500** illustrate the importance of substructural context and can be interpreted as nonadditivity in the SAR. The substitution of the nitrile for an oxime group, **Neq0675** ($pK_i = 7.0$), increased the affinity toward Cz. However, it was not possible to separate the geometric isomers, so **Neq0675** was isolated as a 54/46 E/Z mixture. Therefore, this result needs to be taken with caution. Further studies are on the way in our research group.

We also evaluated isoxazole (**Neq0646**) as possible prodrugs for having an oxime embedded in it. As expected, the absence of a covalent bond formation led to a reduction in affinity. Unsurprisingly, the oxazole **Neq0657** is even less potent than the isoxazole.

Vinyl sulfones have been widely studied for the inhibition of Cz and other cysteine proteases and their antichagasic activities (RENSLO; MCKERROW, 2006). To test the hypothesis that replacement of the sulfonyl with a less strong electron-withdrawing group would lead to reversible inhibition, several vinyl amide and esters were synthesized and tested against Cz.

In general, vinyl esters and amides do not appear to offer any advantage over the more potent nitriles since they are of higher molecular weight. They would be associated with increased risk of poor pharmacokinetics on account of the hydrolytically unstable ester and amide functional groups. The vinyl ester **Neq0638** ($k_{\text{inact}}/K_i = 9.8 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$) is an irreversible inhibitor of cruzain although less potent than the vinyl sulfone K-777 ($k_{\text{inact}}/K_i = 5.1 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$).

Azadipeptide nitriles have been reported to exhibit antitrypanosomal activity and appear useful as activity-based probes due to their potency and proteolytically stability as papain-like cysteine proteases inhibitors (ASAAD et al., 2009; LÖSER et al., 2008; YANG et al., 2012). The azadipeptide nitrile **Neq0677** ($\text{p}K_i = 8.7$) is two orders of magnitude more potent against cruzain than the corresponding nitrile **Neq0500** ($\text{p}K_i = 6.3$). Moved by these promising results, we synthesized two derivatives of **Neq0677** by insertion of the N-tert-butyl-pyrazolic acid in P3 that led to **Neq0689** and **Neq0690**. Different from what we have seen before (see chapter III), results suggest that the binding modes of **Neq0689** and **Neq0690** to cruzain prevent the full benefit of this P3 substituent from being realized.

Azadipeptide nitrile displayed a slow binding kinetic, as reported in the literature (LÖSER et al., 2008), probably due to the presence of the methylamino group as an electron-releasing group. The significant difference in potency between the dipeptide nitrile and their aza analogs could be linked to the imine nitrogen protonation after the rate-determining step.

Biological results were performed for the amastigote form of the *T. cruzi* Y strain infecting U2OS host cells (Figure 29). Results displayed that no selectivity between parasite and the host cell was observed for the azadipeptide nitriles (**Neq0677**, **Neq0689** and **Neq0690**) due to their high intrinsic reactivity and their off-targets liability.

Also, the weak potency of compound **Neq0653** ($\text{pEC}_{50} = 4.4$) should not be correlated with its cruzain inhibition.

At the same time, we evaluated the affinity of **Neq0689** and **Neq690** on the corresponding human cathepsin of cruzain (CatL) and *Leishmania mexicana* CPB. Cysteine protease LmCPB is essential for the virulence of the *Leishmania mexicana* (intracellular protozoan parasite causative agent of the cutaneous form of leishmaniasis), and therefore it is an appealing meaningful target as an antiparasitic therapy. These inhibitors showed an affinity higher than 10 nM for both targets. A 1.3 Å X-ray crystal structure, the first to be reported for LmCPB, was determined for the complex of this enzyme covalently bound to an azadipeptide nitrile (**Neq0690**, Figure 30).

Figure 30. Crystallographic structure of **Neq0690** in complex with LmCPB.)

N°	pK _i (LmCPB)	pK _i (CatL)	pK _i (Cz)
NEQ0689	9.7 (0.04)	9.9 (0.04)	8.8 (0.04)
NEQ0690	9.7 (0.06)	9.3 (0.04)	8.8 (0.04)

(From top to bottom). Schematic 2D binding mode of **Neq0690** in complex with LmCPB, showing the covalent bond between Cys26 and the azanitrile nitrile head group and the stabilization of the formed thioimide by the oxyanion hole. The residues from LmCPB are labeled in black. Hydrogen bonds are represented as red dashed lines structure compound

Neq0690 in complex with LmCPB. Electron density maps ($2F_{\text{obs}} - F_{\text{calc}}$ coefficients in blue) are displayed for the ligand only and are contoured at 1.5σ . The inhibitor displays partial two-fold rotational disorder and is covalently linked to catalytic cysteine (Cys26). The major component is shown above (conformation **I**) while the minor part is shown below (conformation **II**). H-bonds are represented by black dashed lines, and their lengths are displayed in red. Ligand binding subsites, S1, S2 and S3 are also shown along with critical residues. pK_i values for **Neq0689** and **Neq0690** for LmCPB, CatL and Cz. Source: Figures adapted from RIBEIRO, J. F. R.; Crystal structure of *Leishmania mexicana* cysteine protease B in complex with a highly potent azadipeptide nitrile inhibitor. **Scientific Reports**, submitted, jul. 2019.

The mode of binding of **Neq0690** (Figure 30) shows that this inhibitor is covalently bound to the catalytic cysteine (Cys26) by the nucleophilic attack of the thiolate group on the carbon of the nitrile group.

The inhibitor exhibits partial two-fold rotational alternate conformations along the axis of the C-N bond of the chiral carbon atom. The refined occupancy of each conformer is 0.58 and 0.42 for the major (conformation **I**) and minor (conformation **II**) components, respectively. The major part is stabilized by two H-bonds in the S3 subsite between the lone pair-bearing nitrogen on the 1-methylpyrazole ring on the inhibitor, and carboxamide nitrogen of Asn62 side chain, to a single water atom.

Two additional H-bonds between the inhibitor and the enzyme at the S2 subsite are observed. The first occurs between amide nitrogen on the inhibitor to the carbonyl oxygen on Gly67. The second occurs between carbonyl oxygen on the inhibitor to the backbone amide of Gly67. Both of these H-bonds are present in the major and minor orientations of the inhibitor and display only a small shift ($\leq 0.1 \text{ \AA}$) on switching between the two directions. We could speculate that conformation **I**, due to an additional hydrogen bond, led to the difference in affinity between Cz and LmCPB of almost one log unit.

7.3. Conclusion and perspective

In this chapter, we have compared different warheads for Cz inhibition, and we have demonstrated that dipeptide aldehydes and azadipeptide nitriles are significantly more potent inhibitors than dipeptidyl nitriles. Also, we have displayed that the substitution of the nitrile for the oxime group as a warhead improves the Cz affinity and can better the access to the prime region of the catalytic site. Moreover, we have shown how slow binding reversible inhibitors as azadipeptide nitrile are strongly cytotoxic, and therefore, their application is limited as *T. cruzi* killers. In addition, this work should be considered as the first step for long-term research of a warhead with a balanced reactive profile, as they should be sufficiently reactive to form a covalent bond with the cysteine protease in the active site. At the same time, their inherent reactivity should be reduced to a necessary minimum to prevent nonspecific off-target labelling or reaction with any other CPs (CIANNI et al., 2019). A possible strategy could be the application of α -cyano acrylamide as a warhead, as already successfully applied in the near medicinal chemistry field of kinase inhibition (LONSDALE et al., 2017). Or as recently reported for CatK inhibition alkyne was used as latent electrophile warhead (MONS et al., 2019).

8. MATERIAL AND METHODS

8.1. Synthetic chemistry

8.1.1. General consideration

Melting points were determined on a Büchi 510 oil bath apparatus or in a Kofler apparatus and are uncorrected. Infrared spectra were obtained from FT-IR Thermo Scientific Nicolet 380 or FT-IR Bomen Hartman & Braun mod MB-102. Specific rotations ($[\alpha]^T = 100 \alpha/lc$, in $\text{deg mL g}^{-1} \text{ dm}^{-1}$, but reported herein in degrees) were observed at the wavelength 589 nm, the D line of a sodium lamp. T was set to be 25°C. Samples were weight using a precision balance (Sartorius, Model CPA26P) and were fully dissolved in methanol (HPLC grade, Panreac). The rotations were measured using a Digital Polarimeter (P2000, Jasco): α = observed rotation in degrees; l = cell path length of 0.1 decimeter in length; c = concentration in g 100 mL^{-1} . Values were calculated using 5 measurements for each compound.

Reagents, starting materials and solvents were of commercial quality and were used without further purification unless otherwise stated. All syntheses were started with enantiopure amino acids. TLC analysis was carried out on Merck 60 F₂₅₄ silica gel plates and visualized under UV light at 254 nm and 365 nm or by staining solutions such as ninhydrin, ethanolic 5% phosphomolybdic acid solution and aqueous KMnO₄. Purification by column chromatography was carried out on silica gel (Merck 60, particle size 0.040 – 0.063 mm). Preparative column chromatography was carried out on Grace Davison Davisil LC 60A (particle size 0.020 – 0.045 mm) or Merck Geduran Si 60 (particle size 0.063 – 0.200 mm) using an Interchim PuriFlash 430 automated flash chromatography system. The purity of all tested compounds was determined with one of the three (A – C) protocols noted below:

- A) Purity was determined using an LC-MS instrument (AmaZon SL ESI-MS, Shimadzu LC) with a Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μm). The

detection was performed by a UV DAD (200 – 440 nm). An isocrate elution ACN and water as specified, stop time 60 min, flow 0.5 mL/min were used.

- B) Purity was determined using RP-HPLC on a Hewlett Packard 1090 Series II LC with a Phenomenex Luna C18 column (150 x 4.6 mm, 5 μ m) and detection was performed by a UV DAD (200 – 440 nm). Elution was carried out with the following gradient: 0.01 M KH_2PO_4 , pH 2.30 (solvent A), MeOH (solvent B), 40% B to 85% B in 8 min, 85% B for 5 min, 85% to 40% B in 1 min, 40% B for 2 min, stop time 16 min, flow 1.5 mL/min.
- C) Purity was determined using an LC-MS instrument (ABSCIEX API 2000 LC–MS/MS, HPLC Agilent 1100) with a Phenomenex Luna C18 HPLC column (50 x 2.00 mm, 3 μ m) and detection was performed by a UV DAD (200 – 440 nm). Elution was carried out with the following gradient: 0.02 M $\text{NH}_4\text{CH}_3\text{CO}_2$, pH 7.0 (solvent A), MeOH (solvent B) start with 100%, 10% B in 20 min to 100% B, 10 min 100% B, stop time 20 min, flow 0.25 mL/min.

NMR spectra were recorded on Bruker Avance 200 MHz, Bruker Avance 400 MHz and Bruker Avance DRX 500 MHz NMR spectrometers. Chemical shifts are reported in ppm relative to TMS or the residual proton peak of the re-protonated deuterated solvent. The spectra were calibrated against the residual proton peak of the used deuterated solvent. Spin multiplicities are indicated by the following symbols: s (singlet), s br (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), tt (triplet of triplet), q (quartet), sept (septet) and m (multiplet). Standard mass spectra were obtained either as ESI-MS (pos. and/or neg. mode) from a Advion DCMS interface (settings as follows: ESI voltage 3,50 kV, capillary voltage 187 V, source voltage 44 V, capillary temperature 250 °C, desolvation gas temperature 250 °C, gas flow 5 L/min) or by an API 2000 mass spectrometer (electron spray ion source, ABSCIEX, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system.

HRMS spectra were recorded on a Bruker microTOF-Q mass spectrometer connected to a Thermo Scientific Dionex UltiMate 3000 LC via an ESI interface using a Nucleodur C18 Gravity column (50 x 2.0 mm, 3 μ m) or were recorded on Thermo Scientific LTQ Velos Orbitrap, in electrospray ionization (ESI) mode by direct injection.

The synthetic route was developed to optimize the set of substituents to be placed in P1, P2, and P3 that have been defined after the planning and design studies. Due to

the diversity of building blocks, it was necessary to evaluate different methods for the same reaction, aiming at the best yield and preventing racemization.

8.1.2. General procedure for amide synthesis

Method A: The free primary amine (1.0 mmol, 1.0 equiv.) was added to a solution of the carboxylic acid (1.3 mmol, 1.3 equiv.), HATU (490 mg, 1.3 mmol, 1.3 equiv.) and DIPEA (364 mg, 0.45 mL, 2.60 mmol, 2.6 equiv.) in dry DMF (5 mL) under argon atmosphere. The resulting solution was stirred at room temperature for 20 h. The reaction mixture was diluted with ethyl acetate (10 mL) and washed with a saturated NaHCO_3 solution (3 $\times$ 20 mL) and brine (3 $\times$ 20 mL). The organic phase was dried over Na_2SO_4 and evaporated to give a crude residue that was purified by flash column chromatography (CIANNI et al., 2018).

Method B: The free primary amine (1.0 mmol, 1.0 equiv.) was added to a solution of the carboxylic acid (1.3 mmol, 1.3 equiv.), TBTU (410 mg, 1.3 mmol, 1.3 equiv.) and DIPEA (364 mg, 0.45 mL, 2.60 mmol) in dry $\text{DMF}/\text{CH}_2\text{Cl}_2$ (1:1, 10 mL) under argon atmosphere. The resulting solution was stirred at room temperature for 20 h. The reaction mixture was diluted with ethyl acetate (10 mL) and washed with a saturated NaHCO_3 solution (3 $\times$ 20 mL) and brine (3 $\times$ 20 mL). The organic phase was dried over Na_2SO_4 and evaporated to give a crude residue that was purified by flash column chromatography (IVANOV; ZHALNINA; SHISHKOV, 2009).

Method C: Isobutyl chloroformate (790 mg, 0.75 mL, 5.5 mmol, 1.1 equiv) was added dropwise to a solution of Boc-(*R* or *S*) amino acid (5.0 mmol, 1.0 equiv.), DIPEA (1.6 g, 2.28 mL, 13.0 mmol, 2.6 equiv.) in dry DMF (20 mL) under argon atmosphere, at -30 °C and it was stirred for 0.5 h. Then, an aqueous 2 M NH_4Cl solution (294 mg, 2.75 mL, 5.5 mmol, 1.1 equiv.) was added. The resulting solution was stirred at room temperature for 20 h. The reaction mixture was dried under reduced pressure. Ethyl acetate (100 mL) was added and it was washed with a saturated NaHCO_3 solution (3 $\times$ 50 mL) and brine (1 $\times$ 50 mL). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure to give a crude residue that was purified by flash column chromatography (BOWER et al., 2003).

8.1.3. General procedure for removal of the Boc protecting groups

Method A: The Boc-protected amino compound (0.25 mmol, 1.0 equiv.) was treated with an excess of formic acid (2.44 g, 47.9 mmol, 47.9 equiv.) at room temperature. The resulting solution was stirred for 18 h. The reaction mixture was evaporated under reduced pressure to get a yellowish oil. It was treated with an aqueous solution of 1.0 M NaOH until pH 9 was reached. The product was extracted with ethyl acetate (4 × 20 mL) and then washed with brine (1 × 20 mL). The organic phase was evaporated to obtain a colorless oil. The formation of the product was confirmed by TLC (ethyl acetate). The product was used for the next step without further purification (CIANNI et al., 2018).

Method B: To a solution of Boc-protected amino compound (1.0 mmol, 1.0 equiv.) in dry CH₂Cl₂ (3 mL) was added TFA (912 mg, 0.91 mL, 8.00 mmol, 8.0 equiv.) at 0 °C. The mixture was stirred and allowed to reach room temperature within 2 h. The progress of the reaction was monitored by TLC (ethyl acetate). The reaction mixture was evaporated under reduced pressure to eliminate the excess of TFA to get a yellowish solid. The product was used for the next step without further purification (SHENDAGE; FRÖHLICH; HAUFÉ, 2004).

8.1.4. General procedure for dehydration of primary amides to nitriles

Method A: The primary amide (1.0 mmol, 1.0 equiv.) was dissolved in dry DMF (5 mL) at 0 °C. Then, cyanuric chloride (73 mg, 0.4 mmol, 1.1 equiv.) was slowly added to the solution under argon atmosphere. The resulted solution was stirred for 0.5 h. Saturated NaHCO₃ solution (30 mL) was added and it was stirred at room temperature for further 2 h. The product was extracted with ethyl acetate (2 × 50 mL) and then the reunited organic phases were washed with an aqueous solution of 1.0 M KHSO₄ (3 × 20 mL), brine (4 × 30 mL) and dried over Na₂SO₄. The solvent was removed, and the crude product was purified by flash silica gel chromatography (MAETZ; RODRIGUEZ, 1997).

Method B: The primary amide (1.0 mmol, 1.0 equiv.) was dissolved in dry THF (5 mL) and DIPEA (364 mg, 0.45 mL, 2.6 mmol, 2.6 equiv.) was added. Trifluoroacetic anhydride (273 mg, 0.18 mL, 1.30 mmol, 1.3 equiv.) was added over 5 min at 0 °C. The mixture was stirred and allowed to reach roomtemperature within 2 h. Then the reaction was quenched with H₂O (20 mL), THF removed in vacuo, and the product was extracted into ethyl acetate (2 × 50 mL). The organic phase was washed with a solution 1.0 M of KHSO₄ (3 × 20 mL) and with a saturated NaHCO₃ solution (3 × 20 mL) and brine (3 × 20 mL) and dried over Na₂SO₄. The solvent was removed, and the crude product was purified by flash silica gel chromatography (WIKLUND; ROMERO; BERGMAN, 2003).

Method C: The primary amide (1.0 mmol, 1.0 equiv.) was dissolved in dry pyridine (5 mL) at room temperature. Then, *p*-toluenesulfonyl chloride (572 mg, 3.0 mmol, 3.0 equiv.) was added to the solution under argon atmosphere. The resulting solution was stirred for 3 days. Upon addition of saturated NaHCO₃ solution (30 mL), the reaction mixture was stirred at room temperature for 2 h. The solution was dried under reduced pressure. The product was extracted with ethyl acetate (2 × 50 mL) and then the reunited organic phases were washed with a 1.0 M solution of KHSO₄ (2 × 20 mL), brine (4 × 30 mL) and dried over Na₂SO₄. The solvent was removed, and the crude product was purified by flash silica gel chromatography (TSUCHIYA et al., 2013).

8.1.5. General procedure for removal of the benzyl protecting group

Method A: The corresponding protected threonine (1.0 mmol, 1.0 equiv.) was dissolved in abs. ethanol (20 mL) in argon atmosphere. Upon addition of 10% Pd/C, H₂ was bubbled in the solution for 0.5 h. The resulting solution was stirred under H₂ atmosphere for 12 h. The progress of the reaction was monitored by TLC. The solution was filtered on celite two times and dried under reduced pressure to afford the desired product as a colorless wax. The product was used for the next step without further purification(CRISTAU et al., 2003).

Method B: The corresponding protected threonine (1.0 mmol, 1.0 equiv.) was dissolved in dry CH₂Cl₂ (20 mL) under argon atmosphere. Then, DDQ (908 mg, 4.0

mmol, 4.0 equiv) was added and the resulting solution was stirred for 4 days at room temperature. The progress of the reaction was monitored by TLC (ethyl acetate). The reaction was quenched with an aqueous 1.0 M solution of NaHSO_3 (20 mL). Then, CH_2Cl_2 was removed under reduced pressure. The product was extracted with ethyl acetate (2 × 50 mL) and the reunited organic phases were washed with an aqueous solution of 1.0 M KHSO_4 (2 × 20 mL), brine (4 × 30 mL) and dried over Na_2SO_4 . The solvent was removed, and the crude product was purified by flash silica gel chromatography (WRIGHT; YU; SPENCER, 2001).

8.1.6. General procedure for the synthesis of amine esters

The starting amino acid (18 mmol, 1.0 equiv.) was dissolved in MeOH or anhydrous EtOH under argon atmosphere. Then fresh distilled SOCl_2 (19.8 mmol, 1.1 equiv.) was added slowly at 0 °C. The reaction was brought at room temperature and stirred for 4 h. The progress of the reaction was followed by TLC. The solvent was evaporated, and the residue was washed with ethyl acetate (3 × 50 mL) to afford the desired product and it was used for the next step without further purification.

8.1.7. General procedure for imine synthesis

To a solution of amino ester hydrochloride (2.21 mmol, 1.0 equiv.) in dry methanol (5 mL), under argon atmosphere, dried potassium carbonate (4.42 mmol, 2.0 equiv.) and the appropriate trifluoroacetophenone (2.43 mmol, 2.0 equiv.) were added. The mixture was stirred at 50°C for 18 h. Then the reaction was cooled to room temperature and filtered and the solvent was removed under reduced pressure. The collected oil was used in the next step without further purification.

8.1.8. General procedure for imine reduction to amine

Reduction with $\text{Zn}(\text{BH}_4)_2$: To a solution of imine/potassium carboxylate (2.21 mmol, 1.0 equiv.) in ACN (15 mL) and MeOH (1.5 mL), a freshly prepared solution of $\text{Zn}(\text{BH}_4)_2$ in DME (1 mmol/mL, 1.5 mL, 0.7 equiv.) was added at -40/-45 °C. After 3 h at -40/-45 °C, the reaction mixture was allowed to warm until 0°C and 1N HCl was added until pH 2-3. The aqueous mixture was extracted with ethyl acetate (3 × 25 mL); the reunited organic phases were dried on Na_2SO_4 , evaporated under vacuum and purified by flash column chromatography.

Reduction with NaBH_4 : The imine/potassium carboxylate (2.21 mmol, 1.0 equiv.) was suspended in dry THF (25 mL) and MeOH (1.5 mL), stirred at 0 °C and then NaBH_4 (12.15 mmol, 5.6 equiv.) was added. After stirring for 6 h at room temperature, the system was cooled to 0°C and quenched with 2 M HCl solution until stopping the gas release (final pH 2-3). Next, the organic solvent was evaporated under vacuum and the crude extracted with ethyl acetate (3 × 10 mL). The reunited organic phases were dried on Na_2SO_4 , evaporated under vacuum and purified by flash column chromatography.

8.1.9. General procedure for Suzuki cross-coupling reaction

A stream of nitrogen was passed through a suspension of aryl bromide (0.18 mmol, 1.0 equiv.), the opportune boronic acid pinacol ester (0.25 mmol, 1.4 equiv.), 2M Na_2CO_3 (0.26 mL) and DMF (2mL) for 10 min. PdCl_2dppf (8% mol) was then added and the reaction was warmed to 80 °C and stirred under nitrogen for 3h. When the reaction was complete, the mixture was cooled to room temperature and extracted with ethyl acetate (3 x 15 mL). The combined ethyl acetate extracts were dried with sodium sulfate and dried under vacuum to give a residue that was purified by chromatography column to afford the desired compounds.

8.1.10. General procedure for the synthesis of primary sulfonamide

The corresponding free amine (0.2 mmol, 2.0 equiv) was dissolved in CH_2Cl_2 (10 mL) and DIPEA (52 mg, 68 μL , 0.4 mmol, 2.0 equiv.). Under argon atmosphere, this mixture was injected into a solution of the corresponding sulfonyl chloride (0.4 mmol, 2.0 equiv.) in CH_2Cl_2 (10 mL) at 0 °C. The mixture was stirred for 20 h. After evaporation of the solvent, the remaining crude material was partitioned between ethyl acetate (30 mL) and saturated NaHCO_3 solution (30 mL) and extracted. The organic layer was further washed with sat. NaHCO_3 solution (2 $\times$ 30 mL) and brine (3 $\times$ 30 mL). The reunited organic phases were dried on Na_2SO_4 , evaporated under vacuum and purified by flash column chromatography.

8.1.11. Synthesis and characterization of compounds presented in chapter I

Scheme 7. General synthesis of compounds **2–12** and **14**.

Number	Nequimed Code
2	Neq0570
3	Neq0709
4	Neq0710
5	Neq0711
6	Neq0712
7	Neq0717
8	Neq0802
9	Neq0803
10	Neq0804
11	Neq0805
12	Neq0714
14	Neq0710.1

Reagents and conditions: (a) HATU, DIPEA, DMF, rt, 18 h; b) formic acid, rt, 18 h; c) HATU, carboxylic acid, DMF, rt, 18 h. Source: Own author.

Compounds **1** and **13** were synthesized from *N*-(*tert*-Butoxycarbonyl)-L-phenylalanine and 1-amino-1-cyclopropanecarbonitrile following the general procedure for amide synthesis (method A).

(S)-*tert*-butyl (1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate
(**1**)

Yield 92%. White solid. $R_f = 0.9$ (ethyl acetate: *n*-hexane; 7:3). Mp. 146 – 147 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.27 – 7.04 (m, 5H), 4.58 – 4.54 (m, 1H), 3.19 (dd, $J = 13.8$, 5.0 Hz, 1H), 2.88 (dd, $J = 9.5$, 5.0 Hz, 1H), 1.31 (s, 9H), 1.25 – 1.19 (m, 2H), 1.04 – 0.98 (m, 2H). ^{13}C NMR. (125 MHz, CDCl_3) δ 173.06, 155.30, 137.73, 129.35, 128.19, 126.45, 120.80, 78.30, 55.54, 37.36, 28.26, 19.77, 15.80, 15.75. Reference (AVELAR et al., 2015).

(D)-*tert*-butyl (1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate
(**13**)

Yield 92%. White solid. $R_f = 0.9$ (ethyl acetate: *n*-hexane; 7:3). Mp. 141 – 143 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.21 – 7.02 (m, 5H), 4.55– 4.52 (m, 1H), 3.17 (dd, $J = 13.8$, 5.0 Hz, 1H), 2.89 (dd, $J = 9.5$, 5.0 Hz, 1H), 1.31 (s, 9H), 1.22 – 1.15 (m, 2H), 1.03 – 0.96 (m, 2H).

Compounds **2** – **12** and **14** were synthesized in two steps from compounds **1** and **13**, respectively. First, the Boc group was removed (general procedure for removal of the Boc protecting group, method A), and then the free amine was coupled to the selected carboxylic acid following the general procedure for amide synthesis (method A).

(2S)-N-(1-cyanocyclopropyl)-3-phenyl-2-(phenylformamido)propanamide (2)

Yield 70%. White solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 7:3). Mp. 189 – 190°C. NMR ^1H (500 MHz, $\text{DMSO-}d_6$) δ 9.02 (s, 1H), 8.64 (d, J = 8.1 Hz, 1H), 7.82 (d, J = 7.2 Hz, 2H), 7.52 (t, J = 7.3 Hz, 1H), 7.44 (t, J = 7.5 Hz, 2H), 7.30 (d, J = 7.2 Hz, 2H), 7.25 (t, J = 7.5 Hz, 2H), 7.17 (t, J = 7.2 Hz, 1H), 4.60 – 4.57 (m, 1H), 3.17 (dd, J = 13.8, 5.0 Hz, 1H), 2.89 (dd, J = 9.5, 5.0 Hz, 1H), 1.46 – 1.42 (m, 2H), 1.04 – 1.01 (m, 2H). NMR ^{13}C (125 MHz, $\text{DMSO-}d_6$) δ 173.18, 166.71, 138.29, 134.26, 131.81, 129.61, 128.61, 128.55, 127.93, 126.80, 121.18, 55.05, 37.39, 20.19, 16.15, 16.13. HRMS (+) Calc. for $[\text{C}_{20}\text{H}_{20}\text{N}_3\text{O}_2]$ 334.1550, found: 334.1550 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 8.39. Purity: 97.3%. Reference (AVELAR et al., 2015).

(S)-2-chloro-N-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (3)

Yield 80%. White solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 8:2). Mp. 175 – 176 °C. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 8.95 (s, 1H), 8.71 (d, J = 10.0 Hz, 1H), 7.42 – 7.38 (m, 3H), 7.35 (dt, J = 9.5, 3.0 Hz, 1H), 7.28 – 7.17 (m, 5H), 4.57 (m, 1H), 3.06 (dd, J = 13.5, 5.0 Hz, 1H), 2.98 (dd, J = 19.0, 13.5 Hz, 1H), 1.44 – 1.26 (m, 2H), 1.02 – 0.96 (m, 2H). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 172.21, 166.27, 137.53, 136.38, 131.05, 130.21, 129.75, 129.42, 129.17, 128.28, 127.07, 126.62, 120.81, 54.30, 37.22, 19.89, 15.89. FT-IR (KBr, cm^{-1}) 3300.12, 3284.56, 3030.64, 2247.67, 1663.44, 1650.51, 1525.45, 1502.12, 1329.89, 1053.44, 744.62, 698.87. ESI-MS (-) Calc. for $[\text{C}_{20}\text{H}_{18}\text{ClN}_3\text{O}_2]$ 367.83, found: 366.25 $[\text{M}-\text{H}]^-$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 12.01. Purity: 97.2%.

((S)-3-chloro-N-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (4)

Yield 75%. White solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 8:2). Mp. 214 – 215 °C. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 9.03 (s, 1H), 8.83 (d, J = 10.0 Hz, 1H), 7.87 (t, J = 2.6 Hz, 1H), 7.77 (dt, J = 7.5, 1.5 Hz, 1H), 7.59 (ddd, J = 8.0, 2.5, 1.4 Hz, 1H), 7.48 (t, J = 8.0 Hz, 1H), 7.27 – 7.18 (m, 4H), 7.17 – 7.15 (m, 1H), 4.60 – 4.58 (m, 1H), 3.06 (dd, J = 13.5, 5.0 Hz, 1H), 2.98 (dd, J = 19.0, 13.5 Hz, 1H), 1.47 – 1.45 (m, 2H), 1.03 – 0.95 (m, 2H). ^{13}C NMR (125MHz, $\text{DMSO-}d_6$) δ 172.95, 165.31, 138.21, 136.21, 133.49, 131.66, 130.68, 129.57, 128.56, 127.73, 126.84, 126.71, 121.16, 55.12, 37.32, 20.20, 16.14, 16.12. FT-IR (KBr, cm^{-1}) 3302.43, 2924.67, 2243.11, 1672.5, 1649.47, 1537.36, 1525.64, 1319.78, 1076.55, 750.8, 696.32, 686.91. ESI-MS (-) Calc. for $[\text{C}_{20}\text{H}_{18}\text{ClN}_3\text{O}_2]$ 367.83, found: 366.25 $[\text{M-H}]^-$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 14.77. Purity: 94.6%.

(S)-4-chloro-N-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (5)

Yield 75%. White solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 8:2). Mp. 218 – 219 °C. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 9.03 (s, 1H), 8.78 (d, J = 10.0 Hz, 1H), 7.83 (dt, J = 10.5, 2.5 Hz, 2H), 7.52 (dt, J = 10.5, 2.5 Hz, 2H), 7.28 – 7.22 (m, 4H), 7.16 (tt, J = 7, 1.5 Hz, 1H), 4.58 – 4.52 (m, 1H), 3.05 (dd, J = 13.5, 5.0 Hz, 1H), 2.96 (dd, J = 19.0, 13.5 Hz, 1H), 1.45 – 1.39 (m, 2H), 1.01 – 0.94 (m, 2H). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 172.74, 165.38, 137.92, 136.35, 132.69, 129.58, 129.29, 128.43, 128.26, 126.53, 120.87, 54.81, 37.06, 19.89, 15.85, 15.82. FT-IR (KBr, cm^{-1}) 3300.12, 3284.67, 3030.21, 2247.24, 1663.59, 1649.13, 1525.34, 1502.88, 1329.33, 1053.11, 744.67, 698.48. ESI-

MS (-) Calc. for $[C_{20}H_{18}ClN_3O_2]$ 367.83, found: 366.25 $[M-H]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 16.12. Purity: 99.9%.

(S)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-methylbenzamide (**6**)

Yield 73%. White solid. R_f = 0.6 (ethyl acetate: *n*-hexane; 8:2). Mp. 140 – 141 °C 1H NMR (500 MHz, DMSO- d_6) δ 8.87 (s, 1H), 8.39 (d, J = 10.0 Hz, 1H), 7.61 – 7.57 (m, 2H), 7.22 – 7.12 (m, 7H), 4.64 – 4.60 (m, 1H), 3.06 (dd, J = 13.5, 5.0 Hz, 1H), 2.99 (dd, J = 19.0, 13.5 Hz, 1H), 2.33 (s, 3H) 1.37 – 1.31 (m, 2H), 1.06 – 0.96 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 173.00, 166.93, 137.90, 137.67, 133.47, 132.09, 130.08, 129.49, 128.38, 128.31, 126.78, 124.92, 120.71, 78.68, 54.77, 37.56, 21.36, 20.13, 16.13. FT-IR (KBr, cm^{-1}) 3303.67, 3285.43, 3030.11, 2247.27, 1663.44, 1650.21, 1525.8, 1502.62, 1329.47, 1053.28, 744.36, 698.71. ESI-MS (-) Calc. for $[C_{21}H_{21}N_3O_2]$ 347.42, found: 346.31 $[M-H]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 13.5. Purity: 95.8%.

(S)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-hydroxybenzamide (**7**)

Yield 65%. White solid. R_f = 0.3 (ethyl acetate). Mp. 228 – 229 °C. 1H NMR (500 MHz, DMSO- d_6) δ 9.24 – 9.22 (m, 1H), 8.99 (d, J = 10.0 Hz, 1H), 8.11 (d, J = 5.3 Hz, 1H), 7.74 (d, J = 10.0 Hz, 1H), 7.59 (dd, J = 7.5, 3.2 Hz, 1H), 7.46 (d, J = 9.8 Hz, 1H), 7.35 – 7.30 (m, 5H), 5.75 (s, 1H), 4.77 – 4.74 (m, 1H), 3.12 (dd, J = 13.5, 5.0 Hz, 1H), 2.90 (dd, J = 19.0, 13.5 Hz, 1H), 1.53 – 1.51 (m, 2H), 1.13 – 1.09 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.72, 165.11, 156.77, 137.94, 135.98, 133.27, 131.43, 130.46,

129.34, 128.34, 127.50, 126.62, 120.93, 54.88, 37.10, 19.95, 15.90. FT-IR (KBr, cm^{-1}) 3416.66, 3324.13, 3305.56, 2246.94, 1673.25, 1649.72, 1525.16, 1284.44, 1027.1, 706.86. ESI-MS (-) Calc. for $[\text{C}_{20}\text{H}_{19}\text{N}_3\text{O}_3]$ 349.39, found: 348.23 $[\text{M}-\text{H}]^-$. The substance was subjected to semi-preparative chromatography using the following conditions: C18 column (250 $\times$ 10 mm; 10 μm ; 100 Å; LUNA®); isocratic elution with ACN/ water, 30:70; flow rate 2.5 mL/min; injection volume 100 μL ; stock solution of compound was 10.0 mg/mL in mobile phase. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 14.53. Purity: 98.6%.

(S)-N-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-fluorobenzamide
(8)

Yield 75%. White solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 8:2). Mp. 219 – 220 $^{\circ}\text{C}$. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.02 (s, 1H), 8.76 (d, J = 6.5 Hz, 1H), 7.68 – 7.67 (m, 1H), 7.63 – 7.59 (m, 1H), 7.50 (dt, J = 10.0, 7.5 Hz, 1H), 7.40 – 7.35 (m, 1H), 7.30 – 7.23 (m, 4H), 7.16 (tt, J = 6.0, 1.5 Hz, 1H), 4.62 – 4.56 (m, 1H), 3.08 (dd, J = 13.2, 5.1 Hz, 1H), 2.98 (dd, J = 19.0, 12.5 Hz, 1H), 1.47 – 1.44 (m, 2H), 1.04 – 1.01 (m, 2H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 172.95, 165.38, 163.49, 161.06, 138.17, 136.59, 136.53, 130.78, 129.57, 128.55, 126.83, 124.09, 121.15, 55.11, 37.34, 20.17, 16.13. FT-IR (KBr, cm^{-1}) 3271.24, 3064.33, 3026.76, 2297.61, 2237.51, 1676.75, 1664.27, 1587.44, 1541.38, 1523.48, 1485.64, 1435.56, 1377.47, 1325.36, 1300.06, 1288.57, 1271.12, 1224.7, 1215.21, 1180.09, 750.22, 702.98. ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{18}\text{FN}_3\text{O}_2]$ 351.14, found: 352.03 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 12.41. Purity: 96.8%.

(*S*)-3-bromo-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (**9**)

Yield 72%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 8:2). Mp. 229 – 230 °C. ^1H NMR (500 MHz, DMSO- d_6) δ 9.05 (s, 1H), 8.86 (d, J = 8.0 Hz, 1H), 8.04 (t, J = 1.5 Hz, 1H), 7.84 – 7.82 (m, 1H), 7.76 – 7.74 (m, 1H), 7.46 (t, J = 8.0 Hz, 1H), 7.32 – 7.26 (m, 4H), 7.21 (tt, J = 6.0, 1.5 Hz, 1H), 4.64 – 4.60 (m, 1H), 3.15 (dd, J = 13.2, 5.1 Hz, 1H), 3.01 (dd, J = 19.0, 12.5 Hz, 1H), 1.50 – 1.47 (m, 2H), 1.08 – 1.03 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.95, 165.23, 138.19, 136.41, 134.54, 130.94, 130.60, 129.57, 128.56, 128.01, 127.09, 126.84, 121.97, 121.16, 55.11, 37.32, 20.18, 16.12. FT-IR (KBr, cm^{-1}) 3302.04, 3029.21, 3024.23, 2243.54, 1672.86, 1649.28, 1539.01, 1525.6, 1377.43, 1319.11, 1303.56, 1219.81, 1064.77, 896.55, 848.14, 746.33, 700.88, 684.95. ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{18}\text{BrN}_3\text{O}_2]$ 412.29, found: 413.50 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 18.33. Purity: 99.5%.

(*S*)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-iodobenzamide (**10**)

Yield 86%. White solid. R_f = 0.8 (ethyl acetate: *n*-hexane; 8:2). Mp. 205 – 207 °C. ^1H NMR (500 MHz, DMSO- d_6) δ 9.00 (s, 1H), 8.78 (d, J = 8.0 Hz, 1H), 8.17 (t, J = 1.5 Hz, 1H), 7.88 – 7.86 (m, 1H), 7.28 – 7.22 (m, 5H), 7.16 (dt, J = 7.0, 2.0 Hz, 1H), 4.60 – 4.55 (m, 1H), 3.05 (dd, J = 15.0, 5.0 Hz, 1H), 2.96 (dd, J = 14.0, 10.0 Hz, 1H), 1.47 – 1.42 (m, 2H), 1.04 – 1.00 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.97, 165.23, 140.32, 138.19, 136.34, 136.26, 130.85, 129.57, 128.55, 127.41, 126.83, 121.16, 94.96, 55.06, 37.32, 20.18, 16.12. FT-IR (KBr, cm^{-1}) 3286.55, 3029.03, 3022.13, 2241.91, 1672.16, 1649.55, 1522.71, 1317.97, 1284.88, 1299.03, 1219.16, 1066.1,

883.41, 792.65, 744.55, 684.09. ESI-MS (+) Calc. for $[C_{20}H_{18}N_3O_2]$ 460.39, found: 461.32 $[M+H]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 22.26. Purity: 99.1%.

(S)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(trifluoromethyl)benzamide (**11**)

Yield 62%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 7:3). Mp. 193 – 195 °C. 1H NMR (500 MHz, DMSO- d_6) δ 9.04 (s, 1H), 8.98 (d, J = 8.0 Hz, 1H), 8.15 (s, 1H), 8.10 (d, J = 8.0 Hz, 1H), 7.90 (d, J = 7.5 Hz, 1H), 7.70 (t, J = 8.0 Hz, 1H), 7.29 – 7.22 (m, 4H), 7.19 (t, J = 7.5 Hz, 1H), 4.64 – 4.60 (m, 1H), 3.09 (dd, J = 13.5, 5.0 Hz, 1H), 2.98 (dd, J = 13.5, 10.0 Hz, 1H), 1.47 – 1.41 (m, 2H), 1.04 – 1.03 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.91, 165.32, 138.13, 135.13, 132.06, 130.01, 129.58, 129.33, 128.56, 128.39, 126.86, 125.51, 124.57, 121.15, 55.12, 37.37, 20.19, 16.14. FT-IR (KBr, cm^{-1}) 3273.11, 3025.79, 2296.1, 2243.78, 1683.64, 1674.09, 1645.19, 1539.33, 1489.13, 1440.91, 1325.58, 1303.14, 1282.86, 1170.12, 1124.32, 1097.06, 1072.51, 748.66, 696.96, 684.37. ESI-MS (+) Calc. for $[C_{21}H_{18}F_3N_3O_2]$ 401.14, found: 402.15 $[M+H]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 14.75. Purity: 97.9%.

(S)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)nicotinamide (**12**)

Yield 65%. White solid. R_f = 0.3 (ethyl acetate). Mp. 180 – 181 °C. 1H NMR (500 MHz, DMSO- d_6) δ 9.11 (s, 1H), 8.97 – 8.94 (m, 2H), 8.70 (dd, J = 10.5, 5.5 Hz, 1H), 8.15 (dt, J = 10.5, 5.5 Hz, 2H), 7.49 (ddd, J = 15.5, 10.5, 5.0 Hz, 1H), 7.29 – 7.20 (m, 4H), 7.18 – 7.12 (m, 1H), 4.62 (m, 1H), 3.11 (dd, J = 13.5, 5.0 Hz, 1H), 3.10 (dd, J = 19.0, 13.5 Hz, 1H), 1.47 (m, 2H), 1.05 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.91, 165.31, 152.40, 149.04, 138.11, 135.61, 129.80, 129.60, 128.55, 126.83, 123.77, 121.16, 55.07, 39.77, 37.40, 20.19, 16.14, 16.09. FT-IR (KBr, cm^{-1}) 3561.30, 3332.46, 2246.20,

1674.28, 1649.50, 1535.40, 1524.40, 1484.95, 1027.14, 705.0. ESI-MS (-) Calc. for $[C_{19}H_{18}N_4O_2]$ 401.14, found: 333.21 $[M-H]^-$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 8.27. Purity: 98.03%.

((R)-3-chloro-N-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (14)

Yield 68%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 7:3) Mp. 210 – 211 °C. 1H NMR (500 MHz, DMSO- d_6) δ 9.02 (s, 1H), 8.83 (d, J = 10.0 Hz, 1H), 7.87 (t, J = 2.5 Hz, 1H), 7.77 (dt, J = 10.0, 5.0 Hz 1H), 7.60 – 7.58 (m, 1H), 7.48 (t, J = 10.0 Hz, 1H), 7.29 – 7.25 (m, 3H), 7.23 – 7.18 (m, 2H), 4.62 – 4.57 (m, 1H), 3.02 (dd, J = 14.0, 5.5 Hz, 1H), 2.69 (dd, J = 19.0, 13.5 Hz, 1H), 1.47 – 1.44 (m, 2H), 1.04 – 1.01 (m, 2H). ^{13}C NMR (125MHz, DMSO- d_6) δ 172.95, 165.31, 138.21, 136.21, 133.49, 131.66, 130.68, 129.57, 128.56, 127.73, 126.84, 126.71, 121.16, 55.12, 37.32, 20.20, 16.14, 16.12. FT-IR (KBr, cm^{-1}) 3302.48, 2924.86, 2243.21, 1672.31, 1649.95, 1537.06, 1525.49, 1319.15, 1076.04, 750.5, 696.16, 686.12. ESI-MS (-) Calc. for $[C_{20}H_{18}ClN_3O_2]$ 367.83, found: 366.25 $[M-H]^-$. HPLC (protocol A, 35:65 ACN: water): t_R (min) = 37.35. Purity: 97.95%

8.1.12. Synthesis and characterization of compounds presented in chapter II

Scheme 8. General synthesis of compounds **2**, **16** and **18**.

Reagents and conditions: a) HATU, DIPEA, DMF, rt, 18h; b) formic acid, rt, 18h; c) HATU, carboxylic acid, DMF, rt, 18h. Source: Own author.

Synthesis and characterization of compound **1** and **2** have been already reported above.

Compounds **15** and **17** were synthesized from *N*-(*tert*-butoxycarbonyl)-L-phenylalanine and 2-aminoacetonitrile or 2-amino-2-methylpropanenitrile following the general procedure for amide synthesis (method A).

(S)-*tert*-butyl (1-((cyanomethyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**15**)

Yield 89%. White solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 5:5). Mp. 125 – 127 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 8.39 (t, J = 5.0 Hz, 1H), 7.30 – 7.15 (m, 5H), 4.18 – 4.13 (m, 1H), 4.11 (d, J = 5.6 Hz, 2H), 2.99 (dd, J = 13.8, 5.0 Hz, 1H), 2.80 (dd, J = 13.8, 9.5 Hz, 1H), 1.31 (s, 9H). Reference (AVELAR et al., 2015).

(S)-*tert*-butyl (1-((2-cyanopropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**17**)

Yield 85%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 5:5). Mp. 120 – 123 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.33 – 7.16 (m, 5H), 4.15 – 4.13 (m, 1H), 3.05 (dd, J = 13.8, 5.0 Hz, 1H), 2.94 (dd, J = 13.8, 9.5 Hz, 1H), 1.53 (s, 6H), (s, 9H). Reference (AVELAR et al., 2015).

Compounds **16** and **18** were synthesized in two steps from compounds **15** and **17**, respectively. First, the Boc group was removed (procedure A), and then the free amine was coupled to the carboxylic acid following the general procedure for amide synthesis (method A).

(S)-*N*-(1-((cyanomethyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (**16**)

Yield 88%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 8:2). Mp. 170 – 171 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.02 (s, 1H), 8.64 (d, J = 8.1 Hz, 1H), 7.82 (d, J = 7.2 Hz, 2H), 7.52 (t, J = 7.3 Hz, 1H), 7.44 (t, J = 7.5 Hz, 2H), 7.30 (d, J = 7.2 Hz, 2H), 7.25 (t, J = 7.5 Hz, 2H), 7.17 (t, J = 7.2 Hz, 1H), 4.60 – 4.58 (m, 1H), 4.11 (d, J = 5.6 Hz, 2H), 3.05 (dd, J = 13.8, 5.0 Hz, 1H), 2.89 (dd, J = 9.5, 5.0 Hz, 1H). ESI-MS (+) Calc. for $[\text{C}_{18}\text{H}_{17}\text{N}_3\text{O}_2]$ 307.13, found: 308.21 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 11.7. Purity: 98.95%. Reference (AVELAR et al., 2015).

(*S*)-*N*-(1-((2-cyanopropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (**18**)

Yield 78%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 8:2). Mp. 130 – 133 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.05 (s, 1H), 8.68 (d, J = 8.1 Hz, 1H), 7.86 (d, J = 7.2 Hz, 2H), 7.55 (t, J = 7.3 Hz, 1H), 7.44 (t, J = 7.5 Hz, 2H), 7.30 (d, J = 7.2 Hz, 2H), 7.25 (t, J = 7.5 Hz, 2H), 7.17 (t, J = 7.2 Hz, 1H), 4.55 – 4.54 (m, 1H), 3.08 (dd, J = 13.8, 5.0 Hz, 1H), 2.89 (dd, J = 9.5, 5.0 Hz, 1H), 1.58 (s, 6H). ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{22}\text{N}_3\text{O}_2]$ 336.17, found: 338.32 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 11.34. Purity: 95.95%. Reference (AVELAR et al., 2015).

8.1.13. Synthesis and characterization of compounds presented in chapter III

Scheme 9. General synthesis of compounds **2** and **24 – 36**.

Reagents and conditions: a) HATU, DIPEA, 1-amino-1-cyclopropanecarbonitrile, DMF, rt, 18h; b) formic acid, rt, 18h; c) HATU or TBTU, DIPEA, carboxylic acid, DMF, rt, 18h; d) DDQ, CH_2Cl_2 , rt, 18 h. Source: Own author.

Scheme 10. General synthesis of compounds **67–77** and **82–86**.

Reagents and conditions: a) Isobutyl chloroformate, NH_4Cl 2M, DIPEA, DMF, 0 °C to rt, 20 h; b) TFA, CH_2Cl_2 , 0 °C to rt, 2 h; c) HATU, DIPEA, Boc-AA-OH, DMF, rt, 18 h; d) TFA, CH_2Cl_2 , 0 °C to rt, 2 h; e) TBTU, DIPEA, 3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxylic acid, DMF/ CH_2Cl_2 , rt, 18 h; f) Cyanuric chloride, DMF, 0 °C to rt, 0.5 h; g) H_2 (1 atm), Pd/C, rt, 18 h; h) *p*-TOSCl, Py, rt, 3-5 days; i) TFAA, DIPEA, THF, 0 °C to rt, 2 h. Source: Own author.

Table 9. List of Nequimed code for compounds described in chapter III.

Scheme 9		Scheme 10	
Number	Nequimed Code	Number	Nequimed Code
2	Neq0570	67	Neq0937
24	Neq0865	68	Neq0866.1
25	Neq0543	69	Neq0940
26	Neq0533.1	70	Neq0952
27	Neq0945	71	Neq0953
28	Neq0569	72	Neq0954
29	Neq0956	73	Neq0921
30	Neq0719	74	Neq0938
31	Neq0775	75	Neq0922
32	Neq0708	76	Neq0939
33	Neq0777	77	Neq0877
34	Neq0955	82	Neq0941
35	Neq0958	83	Neq0943
36	Neq0957	84	Neq0942
		85	Neq0944
		86	Neq0948

Source: Own author.

Compounds **1** and **19 – 23** were synthesized from the selected amino acid and 1-amino-1-cyclopropanecarbonitrile following the general procedure for amide synthesis (method A). Synthesis and characterization of compound **1** have been already reported above.

(S)-*tert*-Butyl (3-(3-chlorophenyl)-1-((1-cyanocyclopropyl)amino)-1-oxopropan-2-yl)carbamate (**19**)

Yield 83%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 6:4). Mp. 146 – 147 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.26 – 7.25 (m, 1H), 7.23 – 7.21 (m, 1H), 7.08 – 7.03 (m, 2H), 4.27 – 4.24 (m, 1H), 3.10 (dd, J = 13.8, 5.0 Hz, 1H), 2.83 (dd, J = 9.5, 5.0 Hz, 1H), 1.52 – 1.44 (m, 2H), 1.41 (s, 9H), 1.13 – 1.05 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) 172.02, 155.61, 138.23, 134.32, 129.29, 127.49, 127.25, 119.48, 80.55, 55.28, 37.87, 28.18, 20.14, 16.68, 16.56. ESI-MS (+) Calc. for $[\text{C}_{18}\text{H}_{22}\text{ClN}_3\text{O}_3]$ 363.83, found: 364.3 $[\text{M}+\text{H}]^+$.

(*S*)-*tert*-Butyl (1-((1-cyanocyclopropyl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate (**20**)

Yield 75%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 4:6). Mp. 134 – 135 °C. ^1H NMR (200 MHz, CD_3OD) δ 8.45 (d, J = 4.9 Hz, 2H), 7.34 (d, J = 5.8 Hz, 2H), 4.36 – 4.23 (m, 1H), 2.96 (dd, J = 14.0, 5.0 Hz, 2H), 1.53 – 1.46 (m, 1H), 1.38 (s, 9H), 1.20 – 1.14 (m, 2H). ^{13}C NMR (50 MHz, CDCl_3) δ 174.74, 149.95, 149.15, 126.52, 121.09, 80.79, 56.03, 38.88, 38.31, 28.55, 21.21, 17.05. ESI-MS (+) Calc. for $[\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}_3]$ 330.38, found: 331.2 $[\text{M}+\text{H}]^+$.

(*S*)-*tert*-Butyl (1-((1-cyanocyclopropyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (**21**)

Yield 61%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 4:6). Mp. 162 – 164 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.76 (s, 1H), 6.86 (d, J = 7.9 Hz, 1H), 3.86 (dt, J = 8.7, 5.5 Hz, 1H), 1.55 – 1.54 (m, J = 6.6 Hz, 1H), 1.46 – 1.42 (m, 2H), 1.37 – 1.42 (m, 2H), 1.36 (s, 9H), 1.07 (dd, J = 7.7, 5.3 Hz, 2H), 0.84 (2d, J = 6.6 Hz, 6H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 174.17, 155.50, 120.92, 78.22, 52.58, 40.49, 28.31, 24.38, 23.01, 21.66, 19.91, 15.87, 15.75. ESI-MS (+) Calc. for $[\text{C}_{15}\text{H}_{25}\text{N}_3\text{O}_3]$ 295.37, found: 318.3 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((2*S*,3*R*)-3-(benzyloxy)-1-((1-cyanocyclopropyl)amino)-1-oxobutan-2-yl)carbamate (**22**)

Yield 89%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 4:6). Mp. 88 – 90 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.36 – 7.32 (m, 5H), 4.78 – 4.60 (m, 3H), 4.22 – 4.12 (m, 1H), 1.57 – 1.44 (m, 2H), 1.30 – 1.27 (m, 9H), 1.17 – 1.13 (m, 5H). ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{27}\text{N}_3\text{O}_4]$ 373.44, found: 396.4 $[\text{M}+\text{Na}]^+$.

(*S*)-*tert*-butyl (1-((1-cyanocyclopropyl)amino)-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl)carbamate (**23**)

Yield 59%. Yellowish oil. R_f = 0.7 (ethyl acetate: *n*-hexane; 9:1). ^1H NMR (200 MHz, CDCl_3) δ 8.10 (s, 1H), 7.66 – 7.31 (m, 4H), 4.92 – 4.88 (m, 1H), 3.22 – 3.02 (m, 2H), 1.57 – 1.44 (m, 2H), 1.17 – 1.13 (m, 2H). ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{24}\text{N}_4\text{O}_4]$ 368.43, found: 369.32 $[\text{M}+\text{H}]^+$. Reference (AVELAR et al., 2015)

Compounds **2** and **24** – **35** were synthesized in two steps from compounds **1**, **19** – **23**. First, the Boc group was removed (method A for removal of the Boc protecting group), and then the free amine was coupled to the selected carboxylic acid following the general procedure for amide synthesis (method A or B, as indicated). Synthesis and characterization of compounds **2** has been already reported above.

(*S*)-*N*-(3-(3-Chlorophenyl)-1-((1-cyanocyclopropyl)amino)-1-oxopropan-2-yl)benzamide (**24**)

Method A. Yield 86%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 6:4). Mp. 213 – 215 °C. ^1H NMR (500 MHz, DMSO- d_6) δ 9.04 (s, 1H), 8.67 (d, J = 8.1 Hz, 1H), 7.84 – 7.83 (m, 2H), 7.56 – 7.54 (m, 1H), 7.53 (t, J = 7.7 Hz, 2H), 7.41 (s, 1H), 7.32 – 7.25 (m, 3H), 4.65 – 4.60 (m, 1H), 3.09 (dd, J = 13.6, 5.0 Hz, 1H), 3.02 (dd, J = 15.2, 5.0 Hz, 1H), 1.51 – 1.49 (m, 2H), 1.12 – 1.06 (m, 2H). ^{13}C NMR (125 MHz, DMSO- d_6) δ 172.57, 166.48, 140.62, 132.82, 131.52, 130.04, 129.18, 128.31, 128.05, 127.58, 126.50, 120.81, 54.47, 36.60, 19.90, 15.82. HRMS (+) Calc. for $[\text{C}_{20}\text{H}_{19}\text{ClN}_3\text{O}_2]^+$ 368.11658, found: 368.11615 $[\text{M}+\text{H}]^+$. HPLC (protocol C): t_R (min) = 10.29. Purity: 99.6%.

(*S*)-3-(*tert*-Butyl)-*N*-(3-(3-chlorophenyl)-1-((1-cyanocyclopropyl)amino)-1-oxopropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**25**)

Method B. Yield 72%. Yellowish solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 5:5). Mp. 152 – 154 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.03 (s, 1H), 8.59 (d, J = 8.4 Hz, 1H), 7.39 (s, 1H), 7.31 – 7.24 (m, 3H), 6.79 (s, 1H), 4.57 – 4.53 (m, 1H), 3.88 (s, 3H), 3.08 (dd, J = 13.6, 5.1 Hz, 1H), 2.94 (dd, J = 13.6, 10.2 Hz, 1H), 1.50 – 1.47 (m, 2H), 1.28 (s, 9H), 1.10 – 1.05 (m, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.10, 159.46, 158.70, 140.26, 134.95, 132.64, 129.94, 129.15, 127.85, 126.39, 120.63, 103.82, 55.21, 36.38, 31.58, 30.36, 19.75, 15.67, 15.61. HRMS (+) Calc. for $[\text{C}_{22}\text{H}_{27}\text{ClN}_5\text{O}_2]^+$ 428.18533, found: 428.1864 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 11.17. Purity: 98.3%.

(*S*)-3-(*tert*-butyl)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**26**)

Method B. Yield 78%. White solid. R_f = 0.8 (ethyl acetate: *n*-hexane; 8:2). Mp. 175 – 177 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.59 (s, 1H), 7.32 – 7.22 (m, 5H), 6.47 (s, 1H), 4.88 – 4.85 (m, 1H), 4.01 (s, 3H), 3.25 – 3.08 (m, 2H), 1.45 – 1.43 (m, 2H), 1.29 (s, 9H), 1.04 – 1.00 (m, 2H). ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{27}\text{N}_5\text{O}_2]$ 393.48, found: 394.18 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 7.99. Purity: 97.9%. Reference (AVELAR et al., 2015).

(*S*)-3-(*tert*-Butyl)-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**27**)

Method B. Yield 56%. Yellowish oil. R_f = 0.4 (ethyl acetate: *n*-hexane; 5:5). ^1H NMR (200 MHz, CD_3OD) δ 8.48 – 8.46 (m, 2H), 7.43 (d, J = 4.9 Hz, 2H), 6.69 (s, 1H), 4.86 – 4.78 (m, 1H), 3.95 (s, 3H), 3.04 – 3.02 (m, 1H), 2.89 – 2.84 (m, 1H), 1.56 – 1.50 (m, 2H), 1.31 (d, J = 4.3 Hz, 9H), 1.22 – 1.18 (m, 2H). ^{13}C NMR (50 MHz, CD_3OD) δ 172.80, 160.35, 160.05, 148.62, 147.59, 134.80, 125.00, 119.66, 103.60, 53.18, 37.37, 36.45, 31.42, 29.44, 19.92, 15.60, 15.28. FT-IR (KBr, cm^{-1}) 3297.16, 2966.17, 2242.31, 1670.63, 1601.36, 1531.90, 1425.65, 1352.10, 1278.55, 1241.77, 1049.72, 988.42, 808.63, 755.51, 722.82, 511.19, 506.24, 489.90. HRMS (+) Calc. for $[\text{C}_{21}\text{H}_{27}\text{N}_6\text{O}_2]^+$ 395.21955, found: 395.21973 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 3.68. Purity: 94.7%.

(S)-3-(*tert*-butyl)-*N*-(1-((1-cyanocyclopropyl)amino)-4-methyl-1-oxopentan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**28**)

Method B. Yield 73%. Yellowish solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 9:1). Mp. 175 – 177 °C ^1H NMR (200 MHz, CD_3OD) δ 6.78 (s, 1H), 4.58 – 4.50 (m, 1H), 4.04 (s, 3H), 1.88 – 1.45 (m, 5H), 1.30 (s, 9H), 1.27 – 1.20 (m, 2H), 1.02 – 0.95 (m, 2H). ESI-MS (+) Calc. for $[\text{C}_{19}\text{H}_{29}\text{N}_5\text{O}_2]$ 359.47, found: 382.32 $[\text{M}+\text{Na}]^+$. HPLC (protocol B): t_R (min) = 8.05. Purity > 99.9%. Reference (AVELAR et al., 2015)

N-((2*S*,3*R*)-3-(Benzyloxy)-1-((1-cyanocyclopropyl)amino)-1-oxobutan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**29**)

Method B. Yield 53%. Yellowish solid. R_f = 0.4 (ethyl acetate: *n*-hexane; 6:4). Mp. 100 – 101 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.36 – 7.32 (m, 5H), 6.45 (s, 1H), 4.76 – 4.62 (m, 3H), 4.22 – 4.12 (m, 1H), 4.09 (s, 3H), 1.57 – 1.44 (m, 2H), 1.30 – 1.27 (m, 9H), 1.20 – 1.13 (m, 5H). ^{13}C NMR (50 MHz, CDCl_3) δ 170.36, 160.18, 159.74, 137.15, 134.06, 128.48, 127.98, 127.68, 119.25, 103.18, 74.03, 71.48, 54.81, 38.38, 31.66, 30.16, 28.68, 20.24, 16.91, 15.85. FT-IR (KBr, cm^{-1}) 3288.99, 2953.92, 2917.14, 2214.31, 1646.31, 1589.10, 1495.12, 1049.31, 1294.89, 1200.91, 1033.37, 922.51, 755.51, 681.95. HRMS (+) Calc. for $[\text{C}_{24}\text{H}_{32}\text{N}_5\text{O}_3]^+$ 437.25051, found: 438.25102 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 8.77. Purity: 97.0%.

(S)-7-Chloro-*N*-(1-((1-cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)quinoline-4-carboxamide (**30**)

Method A. Yield 74%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 8:2). Mp. 260 – 262 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.15 (d, J = 10.2 Hz, 1H), 9.13 (s, 1H), 8.97 (d, J = 10.2 Hz, 1H), 8.10 (d, J = 2.1 Hz, 1H), 7.72 (d, J = 15 Hz, 1H), 7.56 (dd, J = 15.1, 10.2 Hz, 1H), 7.44 (d, J = 10.2 Hz, 1H), 7.28 – 7.24 (m, 5H), 4.75 – 4.73 (m, 1H), 3.11 (dd, J = 13.8, 5.0 Hz, 1H), 2.87 (dd, J = 9.5, 5.0 Hz, 1H), 1.51 – 1.49 (m, 2H), 1.09 – 1.07 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.34, 166.33, 151.74, 148.36, 141.88, 137.63, 134.53, 129.45, 128.42, 128.00, 127.86, 126.74, 122.89, 120.91, 119.69, 54.56, 37.26, 20.02, 15.89. FT-IR (KBr, cm^{-1}) 3254.05, 2926.14, 2247.17, 1672.36, 1668.36, 1523.83, 846.79, 831.36. HRMS (+) Calc. for $[\text{C}_{23}\text{H}_{20}\text{ClN}_4\text{O}_2]^+$ 418.12748, found: 419.12813 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 17.32. Purity: 99.9%.

(S)-7-Chloro-*N*-(1-((1-cyanocyclopropyl)amino)-4-methyl-1-oxopentan-2-yl)quinoline-4-carboxamide (**31**)

Method A. Yield 68%. White solid. R_f = 0.7 (ethyl acetate). Mp. 169 – 170 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.10 (s, 1H), 9.05 (s, 1H), 9.02 (d, J = 5.5 Hz, 1H), 8.20 (d, J = 11.5 Hz, 1H), 8.15 (d, J = 2.5 Hz, 1H), 7.72 (dd, J = 11.5, 2.5 Hz, 1H), 7.60 (d, J = 5.5 Hz, 1H), 4.48 – 4.46 (m, 1H), 1.70 – 1.63 (m, 3H), 1.52 – 1.48 (m, 2H), 1.19 – 1.14 (m, 2H), 0.92 (2d, J = 10.5 Hz, 6H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 173.63, 166.83, 152.05, 148.70, 142.06, 134.83, 131.26, 128.38, 123.35, 121.21, 120.20, 51.97, 24.87, 23.38, 21.79, 20.34, 16.22, 16.06. FT-IR (KBr, cm^{-1}) 3402.4, 3257.7, 3030.1, 2960.7, 2247.0, 1674.2, 1633.7, 1529.5, 1296.1, 831.31. HRMS (+) Calc. for $[\text{C}_{20}\text{H}_{22}\text{ClN}_4\text{O}_2]^+$ 384.14313, found: 385.14503 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 65:35 ACN: water): t_R (min) = 11.81. Purity: 96.2%.

(*S*)-7-Chloro-*N*-(1-((1-cyanocyclopropyl)amino)-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl)quinoline-4-carboxamide (**32**)

Method A. Yield 49%. Yellowish solid. R_f = 0.3 (ethyl acetate). Mp. 196 – 197 °C. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 10.88 (s, 1H), 9.15 (s, 1H), 9.08 (d, J = 10.2 Hz, 1H), 8.97 (d, J = 4.2 Hz, 1H), 8.10 (d, J = 2.2 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H), 7.54 – 7.52 (m, 1H), 7.46 (d, J = 4.5 Hz, 1H), 7.38 (d, J = 10.2 Hz, 1H), 7.16 (d, J = 2.4 Hz, 1H), 7.09 (t, J = 5.5 Hz, 1H), 7.00 (t, J = 5.8 Hz, 1H), 4.81 – 4.79 (m, 1H), 3.20 (dd, J = 13.8, 5.0 Hz, 1H), 3.05 (dd, J = 9.5, 5.0 Hz, 1H), 1.51 – 1.48 (m, 2H), 1.11 – 1.08 (m, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 173.02, 166.59, 151.94, 148.61, 142.38, 136.57, 134.72, 128.23, 128.05, 128.03, 127.52, 124.56, 123.19, 121.46, 121.26, 119.97, 119.03, 118.76, 111.81, 110.02, 54.26, 27.79, 20.33, 16.20. FT-IR (KBr, cm^{-1}) 3254.05, 2926.14, 2247.17, 1672.36, 1665.34, 1522.83, 831.30, 732.98. HRMS (+) Calc. for $[\text{C}_{25}\text{H}_{21}\text{ClN}_5\text{O}_2]^+$ 458.13838, found: 458.13703 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 50:50 ACN: water): t_R (min) = 18.64. Purity: 99.6%.

(*S*)-3-((1-((1-Cyanocyclopropyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)phenyl benzoate (**33**)

Method A. Yield 89%. White solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 6:4). Mp. 185 – 187 °C. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.05 (s, 1H), 8.79 (d, J = 8.0 Hz, 1H), 8.18 – 8.16 (m, 2H), 7.81 – 7.78 (m, 3H), 7.64 (t, J = 8.5 Hz, 2H), 7.57 (t, J = 8.5 Hz, 2H), 7.50 – 7.48 (m, 1H), 7.31 – 7.23 (m, 4H), 7.18 (tt, J = 7.0, 1.5 Hz, 1H), 4.64 – 4.59 (m, 1H), 3.06 (dd, J = 13.6, 5.0 Hz, 1H), 3.00 (dd, J = 13.5, 8.5 Hz, 1H), 1.49 – 1.45 (m, 2H),

1.06 – 1.01 (m, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 173.04, 165.60, 165.02, 150.88, 138.21, 135.72, 134.66, 130.27, 129.98, 129.57, 129.51, 129.16, 128.57, 126.83, 125.69, 125.53, 121.47, 121.15, 55.10, 37.36, 20.18, 16.15, 16.10, 14.55. FT-IR (KBr, cm^{-1}) 3254.05, 2926.14, 2247.17, 1672.36, 1668.36, 1523.83, 846.79, 831.36. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{24}\text{N}_3\text{O}_4]^+$ 453.17668, found: 454.17800 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 65:35 ACN: water): t_R (min) = 19.62. Purity: 99.9%.

(S)-6-Amino-N-(1-((1-cyanocyclopropyl)amino)-4-methyl-1-oxopentan-2-yl)nicotinamide (**34**)

Method B. Yield 48%. Yellowish solid. R_f = 0.3 (ethyl acetate: methanol; 8:2). Mp. 100 – 101 °C. ^1H NMR (200 MHz, CD_3OD) δ 8.43 (s, 1H), 7.88 (d, J = 8.8, 2.1 Hz, 1H), 6.54 (d, J = 8.9 Hz, 1H), 4.53 – 4.46 (m, 1H), 1.82 – 1.51 (m, 3H), 1.47 – 1.43 (m, 2H), 1.24 – 1.17 (m, 2H), 0.96 – 0.93 (m, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.59, 168.58, 162.86, 149.21, 138.44, 121.42, 119.42, 109.12, 41.60, 26.13, 23.54, 21.98, 21.41, 17.15, 16.78. FT-IR (KBr, cm^{-1}) 3288.99, 2953.92, 2917.14, 2214.31, 1647.63, 1496.06, 1409.33, 1294.89, 1202.33, 1075.33, 1030.81, 922.54, 763.49, 667.21. ESI-MS (+) Calc. for $[\text{C}_{16}\text{H}_{22}\text{N}_5\text{O}_2]^+$ 316.17735, found: 316.17713 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 8.77. Purity: 97.0%.

(S)-N-(1-((1-Cyanocyclopropyl)amino)-4-methyl-1-oxopentan-2-yl)-1H-pyrrolo[2,3-b]pyridine-5-carboxamide (**35**)

Method B. Yield 45%. White solid. R_f = 0.3 (ethyl acetate: methanol; 8:2). Mp. 79 – 80 °C. ^1H NMR (200 MHz, CD_3OD) δ 8.71 (d, J = 1.8 Hz, 1H), 8.47 (d, J = 2.0 Hz, 1H), 7.46 (d, J = 3.5 Hz, 1H), 6.57 (d, J = 3.5 Hz, 1H), 4.59 – 4.57 (m, 1H), 1.91 – 1.56 (m, 3H), 1.54 – 1.49 (m, 2H), 1.29 – 1.25 (m, 2H), 1.01 – 0.89 (m, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 176.50, 169.61, 163.45, 150.38, 143.32, 129.52, 128.65, 123.21, 120.94,

102.66, 53.50, 38.92, 26.18, 23.41, 22.02, 21.25, 17.17, 16.49. FT-IR (KBr, cm^{-1}) 3286.44, 2933.89, 2924.14, 2216.51, 1647.63, 1588.10, 1496.06, 1409.33, 1294.89, 1202.33, 1075.33, 1030.31, 922.54, 763.54, 667.21. ESI-MS (+) Calc. for $[\text{C}_{18}\text{H}_{22}\text{N}_5\text{O}_2]^+$ 340.17753, found: 340.17689 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 5.38. Purity: 99.0%.

3-(tert-Butyl)-N-((2S,3R)-1-((1-cyanocyclopropyl)amino)-3-hydroxy-1-oxobutan-2-yl)-1-methyl-1H-pyrazole-5-carboxamide (36)

Compound **36** was synthesized from compound **29** by removal of the benzyl protecting group under mild conditions (protocol B).

Yield 40%. Yellowish solid. R_f = 0.3 (ethyl acetate). Mp. 201 – 202 °C. ^1H NMR (200 MHz, CD_3OD) δ 6.81 (s, 1H), 4.40 (d, J = 4.4 Hz, 1H), 4.23 – 4.18 (m, 1H), 4.04 (s, 3H), 1.52 – 1.47 (m, 2H), 1.32 (s, 9H), 1.29 – 1.18 (m, 5H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.94, 163.34, 162.74, 137.60, 122.22, 106.16, 69.42, 61.17, 39.91, 34.00, 31.94, 22.41, 21.45, 18.11, 17.83. FT-IR (KBr, cm^{-1}) 3288.89, 2953.92, 2917.14, 2214.31, 1646.31, 1589.10, 1495.12, 1049.31, 1294.89, 1200.91, 1033.97, 922.51, 755.51, 681.95. ESI-MS (-) Calc. for $[\text{C}_{17}\text{H}_{25}\text{N}_5\text{O}_3]^+$ 348.20356, found: 348.20314 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 7.74. Purity: 98.0%.

Compounds **37** – **44** were synthesized from the equivalent amino Boc-protected amino acid following the general procedure for amide synthesis (method C).

(S)-tert-Butyl (1-amino-1-oxo-3-phenylpropan-2-yl)carbamate (37)

Yield 77%. White solid. R_f = 0.6 (ethyl acetate). Mp. 146 – 149 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.26 – 7.16 (m, 5H), 4.44 – 4.41 (m, 1H), 3.14 (dd, J = 13.7, 6.0 Hz, 1H), 2.88 (dd, J = 13.7, 8.1 Hz, 1H), 1.23 (s, 9H). ^{13}C NMR (50 MHz, CD_3OD) δ 173.98,

155.60, 130.73, 124.93, 122.46, 118.22, 80.20, 54.02, 37.73, 27.94. ESI-MS (+) Calc. for $[C_{14}H_{20}N_2O_3]$ 264.31, found: 287.3 $[M+Na]^+$.

(R)-tert-butyl (1-amino-1-oxo-3-phenylpropan-2-yl)carbamate (**38**)

Yield 79%. White solid. R_f = 0.6 (ethyl acetate). Mp. 141 – 142 °C. 1H NMR (200 MHz, CD_3OD) δ 7.24 – 7.17 (m, 5H), 4.42 – 4.37 (m, 1H), 3.14 (dd, J = 13.7, 6.0 Hz, 1H), 2.92 (dd, J = 13.7, 8.1 Hz, 1H), 1.43 (s, 9H). ^{13}C NMR (50 MHz, CD_3OD) δ 173.12, 154.48, 133.36, 122.46, 121.75, 119.38, 83.51, 55.61, 38.38, 28.66. ESI-MS (+) Calc. for $[C_{14}H_{20}N_2O_3]$ 264.31, found: 287.3 $[M+Na]^+$.

(S)-tert-Butyl (1-amino-4-methyl-1-oxopentan-2-yl)carbamate (**39**)

Yield 74%. White solid. R_f = 0.4 (ethyl acetate). Mp. 138 – 141 °C. 1H NMR (200 MHz, $CDCl_3$) δ 6.58 (s br, 1H), 6.13 (s br, 1H), 5.25 – 4.97 (m, 2H), 4.15 (s br, 1H), 1.73 – 1.43 (m, 3H), 1.41 (s, 9H), 0.92 (2d, J = 10.5 Hz, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.34, 155.95, 71.56, 28.57, 28.18, 25.08, 23.21, 19.25. ESI-MS (+) Calc. for $[C_{11}H_{22}N_2O_3]$ 230.30, found: 253.3 $[M+Na]^+$.

(R)-tert-Butyl (1-amino-4-methyl-1-oxopentan-2-yl)carbamate (**40**)

Yield 71%. White solid. R_f = 0.4 (ethyl acetate). Mp. 138 – 141 °C. 1H NMR (200 MHz, $CDCl_3$) δ 6.64 (s br, 1H), 6.21 (s br, 1H), 5.21 – 4.98 (m, 2H), 4.16 (s br, 1H), 1.71 – 1.45 (m, 3H), 1.42 (s, 9H), 0.93 (2d, J = 10.5 Hz, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.11, 155.37, 71.64, 28.61, 28.29, 25.15, 23.34, 19.30. ESI-MS (+) Calc. for $[C_{11}H_{22}N_2O_3]$ 230.30, found: 253.3 $[M+Na]^+$.

(S)-*tert*-Butyl (1-amino-3-(3-chlorophenyl)-1-oxopropan-2-yl)carbamate (**41**)

Yield 74%. White solid. R_f = 0.4 (ethyl acetate). Mp. 111 – 112 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.26 – 7.13 (m, 4H), 6.16 (s, 1H), 5.75 (s, 1H), 5.21 (d, J = 7.8 Hz, 1H), 4.42 – 4.36 (m, 1H), 3.19 – 2.94 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 173.53, 155.16, 138.90, 134.09, 130.04, 129.44, 127.55, 127.11, 80.31, 37.88, 28.16. ESI-MS (+) Calc. for $[\text{C}_{14}\text{H}_{19}\text{ClN}_2\text{O}_3]$ 298.77, found: 321.8 $[\text{M}+\text{Na}]^+$.

(S)-*tert*-Butyl (1-amino-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate (**42**)

Yield 92%. White solid. R_f = 0.2 (ethyl acetate). Mp. 131 – 133 °C. ^1H NMR (200 MHz, CDCl_3) δ 8.42 (d, J = 5.7 Hz, 2H), 7.22 (d, J = 5.9 Hz, 2H), 4.35 – 4.31 (m, 1H), 3.18 (dd, J = 13.7, 6.0 Hz, 1H), 2.81 (dd, J = 13.7, 8.1 Hz, 1H), 1.49–1.33 (s br, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 173.69, 155.86, 148.48, 147.61, 118.42, 80.22, 54.51, 37.77, 27.89. ESI-MS (+) Calc. for $[\text{C}_{13}\text{H}_{19}\text{N}_3\text{O}_3]$ 265.31, found: 266.2 $[\text{M}+\text{H}]^+$.

tert-Butyl ((2*S*,3*R*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)carbamate (**43**)

Yield 65%. White solid. R_f = 0.5 (ethyl acetate). Mp. 145 – 147 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.37 – 7.28 (m, 5H), 6.50 (s, 1H), 5.70 – 5.51 (m, 2H), 4.62 (q, J = 4.6 Hz, 2H), 4.34 – 4.31 (m, 1H), 4.18 – 4.11 (m, 1H), 1.45 (s, 9H), 1.19 (d, J = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.09, 155.66, 137.78, 128.41, 127.82, 127.73, 80.03, 71.61, 57.13, 28.23, 27.84, 18.92. ESI-MS (+) Calc. for $[\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_4]$ 308.37, found: 331.4 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((2*R*,3*S*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)carbamate (**44**)

Yield 68%. White solid. R_f = 0.5 (ethyl acetate). Mp. 143 – 145 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.37 – 7.28 (m, 5H), 6.50 (s, 1H), 5.70 – 5.51 (m, 2H), 4.62 (q, J = 4.6 Hz, 2H), 4.33 (s br, 1H), 4.27 – 4.19 (m, 1H), 1.45 (s, 9H), 1.19 (d, J = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.74, 156.31, 138.44, 129.06, 128.47, 128.38, 80.68, 72.26, 57.78, 28.89, 28.49, 19.57. ESI-MS (+) Calc. for $[\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_4]$ 308.37, found: 331.4 $[\text{M}+\text{Na}]^+$.

Compounds **45** – **55** were synthesized in two steps from their precursors **37** – **44**. After removal of the Boc-protecting group (procedure B), the resulting free amine was coupled to the carboxylic acid following the general procedure for amide synthesis (method A).

tert-Butyl ((*S*)-1-(((*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**45**)

Yield 92%. White solid. R_f = 0.8 (ethyl acetate). Mp. 186 – 188 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.37 – 7.22 (m, 6H), 7.16 – 7.09 (m, 4H), 4.76 – 4.63 (m, 1H), 4.34 – 4.20 (m, 1H), 3.15 – 2.95 (m, 2H), 2.91 – 2.62 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.36, 172.35, 156.09, 136.44, 129.04, 128.35, 128.27, 126.78, 126.64, 79.89, 56.00, 53.78, 37.73, 37.24, 27.84. ESI-MS (+) Calc. for $[\text{C}_{23}\text{H}_{29}\text{N}_3\text{O}_4]$ 411.49, found: 434.3 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((*R*)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**46**)

Yield 86%. White solid. R_f = 0.8 (ethyl acetate). Mp. 185 – 187 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.75 – 7.60 (m, 6H), 7.56 – 7.49 (m, 4H), 5.14 – 5.01 (m, 1H), 4.72 – 4.57 (m, 1H), 3.53 – 3.33 (m, 2H), 3.29 – 3.00 (m, 2H), 1.77 (s, 9H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.85, 172.83, 156.58, 136.93, 129.53, 128.83, 128.76, 127.26, 127.13, 80.38, 56.49, 54.26, 38.22, 37.73, 28.33. ESI-MS (+) Calc. for $[\text{C}_{23}\text{H}_{29}\text{N}_3\text{O}_4]$ 411.49, found: 434.3 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((*S*)-1-amino-4-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**47**)

Yield 83%. White solid. R_f = 0.8 (ethyl acetate). Mp. 164 – 165 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.24 – 7.20 (m, 5H), 4.33 – 4.28 (m, 1H), 4.11 – 4.05 (m, 1H), 3.03 – 2.93 (m, 2H), 1.56 – 1.38 (m, 2H), 1.36 (s, 9H), 1.26 – 1.24 (m, 1H), 0.89 (d, J = 2.6 Hz, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 175.39, 154.59, 143.42, 136.55, 128.99, 128.29, 126.65, 84.91, 72.86, 52.36, 51.47, 27.61, 24.41, 22.20, 21.01. ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{31}\text{N}_3\text{O}_4]$ 377.48, found: 400.5 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((*R*)-1-amino-4-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**48**)

Yield 88%. White solid. R_f = 0.8 (ethyl acetate). Mp. 168 – 170 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.24 – 7.21 (m, 5H), 4.31 – 4.21 (m, 1H), 4.11 – 4.09 (m, 1H), 3.04 – 2.94 (m, 2H), 1.57 – 1.40 (m, 2H), 1.39 (s, 9H), 1.27 – 1.24 (m, 1H), 0.90 (d, J = 2.6 Hz, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 175.80, 155.00, 143.83, 136.96, 129.40, 128.70, 127.06, 85.32, 77.16, 73.27, 52.78, 51.89, 28.02, 24.82, 22.61, 21.42. ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{31}\text{N}_3\text{O}_4]$ 377.48, found: 400.5 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((*S*)-1-amino-3-(3-chlorophenyl)-1-oxopropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**49**)

Yield 78%. White solid. R_f = 0.6 (ethyl acetate). Mp. 148 – 150 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.38 – 7.26 (m, 7H), 7.10 – 7.06 (m, 2H), 6.63 (s br, 1H), 6.39 (s br, 1H), 4.81 – 4.77 (m, 1H), 4.38 – 4.35 (m, 1H), 3.05 – 2.97 (m, 4H), 1.41 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 172.83, 161.82, 138.54, 136.14, 134.46, 133.26, 130.05, 129.51, 129.36, 129.02, 127.69, 127.36, 126.78, 80.95, 62.51, 53.21, 52.76, 38.74, 28.23. ESI-MS (+) Calc. for $[\text{C}_{23}\text{H}_{28}\text{ClN}_3\text{O}_4]$ 445.94, found: 468.8 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((*S*)-1-amino-1-oxo-3-(pyridin-4-yl)propan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**50**)

Yield 71%. Yellowish solid. R_f = 0.4 (ethyl acetate). Mp. 155 – 157 °C. ^1H NMR (200 MHz, CDCl_3) δ 8.63 (d, J = 5.7 Hz, 2H), 7.58 – 7.30 (m, 7H), 4.94 – 4.89 (1H, m), 4.55 – 4.40 (m, 1H), 3.44 (dd, J = 13.8, 5.5 Hz, 2H), 3.28 – 2.93 (m, 2H), 1.60 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 173.27, 172.38, 148.58, 140.95, 136.44, 135.10, 128.97, 128.32, 126.68, 124.97, 99.74, 55.40, 27.73, 23.92, 13.06, 10.47. ESI-MS (+) Calc. for $[\text{C}_{23}\text{H}_{28}\text{ClN}_3\text{O}_4]$ 445.94, found: 468.8 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((2*S*,3*R*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**51**)

Yield 88%. Yellowish solid. R_f = 0.8 (ethyl acetate). Mp. 161 – 163 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.38 – 7.26 (m, 7H), 7.10 – 7.06 (m, 2H), 6.63 (s br, 1H), 6.39 (s br, 1H), 5.65 (s br, 1H), 5.08 (s br, 1H), 4.81 – 4.77 (m, 1H), 4.38 – 4.35 (m, 1H), 3.15 – 2.97 (m, 5H), 1.74 – 1.47 (m, 3H), 1.41 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 172.98, 171.90, 156.38, 137.70, 136.53, 128.99, 128.42, 128.13, 127.64, 127.58, 126.75, 80.30, 74.03, 71.36, 60.44, 38.05, 27.67, 20.33, 15.75. ESI-MS (+) Calc. for $[\text{C}_{25}\text{H}_{33}\text{N}_3\text{O}_5]$ 455.55, found: 478.5 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((2*R*,3*S*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**52**)

Yield 78%. Yellowish solid. R_f = 0.8 (ethyl acetate). Mp. 138 – 139 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.40 – 7.27 (m, 7H), 7.11 – 7.08 (m, 2H), 6.60 (s br, 1H), 6.43 (s br, 1H), 5.61 (s br, 1H), 5.23 (s br, 1H), 4.81 – 4.78 (m, 1H), 4.36 – 4.34 (m, 1H), 3.16 – 2.95 (m, 5H), 1.39 (s, 9H), 1.19 – 1.09 (m, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 173.34, 172.27, 156.74, 138.07, 136.90, 129.35, 128.79, 128.49, 128.01, 127.95, 127.12, 80.66, 74.40, 71.73, 60.81, 38.42, 28.04, 20.70, 16.12. ESI-MS (+) Calc. for $[\text{C}_{25}\text{H}_{33}\text{N}_3\text{O}_5]$ 455.55, found: 478.5 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((2*S*,3*R*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (**53**)

Yield 76%. Yellowish solid. R_f = 0.8 (ethyl acetate). Mp. 88 – 91 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.30 – 7.22 (m, 4H), 7.19 – 7.15 (m, 1H), 6.24 (s br, 1H), 6.04 (s br, 1H), 5.36 (s br, 2H), 4.57 (s, 2H), 4.56 – 4.52 (m, 1H), 4.36 – 4.21 (m, 1H), 4.17 – 4.11 (m, 1H), 1.77 – 1.48 (m, 3H), 1.38 (s, 9H), 1.13 (d, J = 6.4 Hz, 3H), 0.94 – 0.90 (m, 6H). ^{13}C NMR (50 MHz, CDCl_3) δ 172.84, 171.86, 155.98, 137.88, 128.29, 127.66, 127.63, 80.38, 73.89, 71.58, 56.40, 54.01, 40.85, 28.12, 24.74, 22.92, 21.62. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{35}\text{N}_3\text{O}_5]$ 421.53, found: 444.7 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((2*R*,3*S*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (**54**)

Yield 73%. Yellowish solid. R_f = 0.8 (ethyl acetate). Mp. 86 – 88 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.31 – 7.22 (m, 5H), 7.20 – 7.11 (m, 1H), 6.26 (s br, 1H), 6.02 (s br, 1H), 5.36 (s br, 2H), 4.54 (s, 2H), 4.56 – 4.53 (m, 1H), 4.33 – 4.28 (m, 1H), 4.16 – 4.11 (m, 1H), 1.76 – 1.48 (m, 3H), 1.48 (s, 9H), 1.11 (d, J = 6.4 Hz, 3H), 0.96 – 0.90 (m, 6H). ^{13}C NMR (50 MHz, CDCl_3) δ 172.84, 171.86, 155.98, 137.88, 128.29, 127.66, 127.63, 80.38, 73.89, 71.58, 56.40, 54.01, 40.85, 28.12, 24.74, 22.92, 21.62. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{35}\text{N}_3\text{O}_5]$ 421.53, found: 444.7 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((2*S*,3*R*)-1-amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-3-(3-chlorophenyl)-1-oxopropan-2-yl)carbamate (**55**)

Yield 77%. Yellowish solid. R_f = 0.6 (ethyl acetate). Mp. 164 – 165 °C. ^1H NMR (200 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 7.47 – 7.22 (m, 9H), 4.55 – 4.37 (m, 1H), 4.29 – 4.17 (m, 2H), 3.26 (dd, J = 13.9, 5.1 Hz, 1H), 3.07 – 2.96 (m, 1H), 2.92 (s, 2H), 1.44 (s, 9H), 1.27 (d, J = 6.3 Hz, 3H). ^{13}C NMR (50 MHz, CD_3OD) δ 172.84, 171.76, 156.24, 138.90, 137.56, 136.39, 130.04, 128.85, 128.28, 127.99, 127.50, 127.44, 126.61, 80.16, 73.89, 71.22, 60.30, 49.00, 37.92, 27.53, 20.19. ESI-MS (+) Calc. for $[\text{C}_{25}\text{H}_{32}\text{ClN}_3\text{O}_5]$ 489.99, found: 513.1 $[\text{M}+\text{Na}]^+$.

Compounds **56** – **66** were synthesized in two steps from their precursors **45** – **55**. After removal of the Boc-protecting group (method B), the free amine was coupled to the selected carboxylic acid following the general procedure for amide synthesis (method B).

N-((*S*)-1-(((*S*)-1-Amino-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**56**)

Yield 79%. Yellowish solid. $R_f = 0.3$ (ethyl acetate). Mp. 109 – 110 °C. ^1H NMR (200 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 7.32 – 6.96 (m, 10H), 6.65 (s br, 1H), 6.26 (s, 1H), 6.09 (s br, 1H), 4.68 – 4.58 (m, 2H), 3.81 (s, 3H), 2.87 – 2.74 (m, 4H), 1.11 (s, 9H). ^{13}C NMR (50 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 173.37, 165.56, 162.46, 160.20, 159.99, 136.34, 136.16, 134.40, 129.13, 128.39, 126.87, 126.81, 124.46, 103.30, 54.93, 53.91, 38.44, 36.34, 31.69, 31.28, 30.27. ESI-MS (+) Calc. for $[\text{C}_{27}\text{H}_{33}\text{N}_5\text{O}_3]$ 475.58, found: 498.7 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((*R*)-1-Amino-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**57**)

Yield 72%. Yellowish solid. $R_f = 0.4$ (ethyl acetate). Mp. 121 – 122 °C. ^1H NMR (200 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 7.59 – 7.13 (m, 10H), 6.88 (s, 1H), 6.50 (s, 1H), 6.32 (s br, 1H), 4.92 – 4.82 (m, 2H), 4.04 (s, 3H), 3.10 – 2.91 (m, 4H), 1.34 (s, 9H). ^{13}C NMR (50 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 172.94, 165.13, 162.04, 159.77, 159.56, 135.92, 135.74, 133.97, 128.70, 127.97, 126.45, 126.38, 124.04, 102.88, 54.50, 53.48, 38.01, 35.91, 31.26, 30.86, 29.84. ESI-MS (+) Calc. for $[\text{C}_{27}\text{H}_{33}\text{N}_5\text{O}_3]$ 475.58, found: 498.7 $[\text{M}+\text{Na}]^+$.

N-((S)-1-(((S)-1-Amino-4-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(tert-butyl)-1-methyl-1H-pyrazole-5-carboxamide (58)

Yield 85%. Yellowish solid. R_f = 0.3 (ethyl acetate). Mp. 152 – 153 °C. $^1\text{H-NMR}$ (200 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ . 7.42 – 7.25 (m, 5H), 6.81 (s, 1H), 4.77 – 4.65 (m, 1H), 4.36 – 4.08 (m, 1H), 4.08 (s, 3H), 3.22 – 3.18 (m, 2H), 1.89 – 1.46 (m, 3H), 1.40 (s, 9H), 0.88 – 0.80 (m, 6H). ^{13}C NMR (50 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 173.57, 168.11, 162.00, 161.25, 137.23, 135.94, 130.02, 129.31, 127.64, 104.97, 57.07, 49.00, 38.87, 32.59, 31.75, 30.78, 24.80, 23.57, 21.42. ESI-MS (+) Calc. for $[\text{C}_{24}\text{H}_{35}\text{N}_5\text{O}_3]$ 441.57, found: 464.5 $[\text{M}+\text{Na}]^+$.

N-((S)-1-(((R)-1-Amino-4-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(tert-butyl)-1-methyl-1H-pyrazole-5-carboxamide (59)

Yield 72%. Yellowish solid. R_f = 0.3 (ethyl acetate). Mp. 131 – 133 °C. ^1H NMR (200 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$ 10:1) δ 7.45 – 7.28 (s, 5H), 6.83 (s, 1H), 4.75 – 4.68 (m, 1H), 4.35 – 4.14 (m, 1H), 4.09 (s, 3H), 3.25 – 3.21 (m, 2H), 1.85 – 1.44 (m, 3H), 1.42 (s, 9H), 0.88 – 0.83 (m, 6H). ^{13}C NMR (50 MHz, CDCl_3) δ 176.49, 171.02, 164.92, 164.17, 140.15, 138.86, 132.94, 132.23, 130.56, 107.89, 76.35, 59.99, 41.79, 35.51, 34.67, 33.70, 27.72, 26.49, 24.34. ESI-MS (+) Calc. for $[\text{C}_{24}\text{H}_{35}\text{N}_5\text{O}_3]$ 441.57, found: 464.5 $[\text{M}+\text{Na}]^+$.

tert-Butyl ((*S*)-1-(((*S*)-1-amino-3-(3-chlorophenyl)-1-oxopropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (**60**)

Yield 80%. Yellowish solid. R_f = 0.2 (ethyl acetate). Mp. 101 – 103 °C. ^1H NMR (200 MHz, CDCl_3 : CD_3OD 10:1) δ . 7.58 – 6.99 (m, 9H), 6.47 (s, 1H), 4.77 – 4.62 (m, 2H), 4.04 (s, 3H), 3.39 – 2.91 (m, 4H), 1.34 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3 : CD_3OD 10:1) δ 172.23, 172.21, 161.25, 161.03, 137.40, 136.96, 135.83, 135.15, 130.68, 129.87, 129.23, 128.42, 128.30, 127.69, 117.62, 104.55, 55.12, 49.00, 42.41, 38.76, 38.62, 32.51, 30.78. ESI-MS (+) Calc. for $[\text{C}_{27}\text{H}_{32}\text{ClN}_5\text{O}_3]$ 510.03, found: 532.9 $[\text{M}+\text{Na}]^+$.

N-(((*S*)-1-(((*S*)-1-Amino-1-oxo-3-(pyridin-4-yl)propan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1H-pyrazole-5-carboxamide (**61**)

Yield 66%. Yellowish oil. R_f = 0.2 (ethyl acetate). ^1H NMR (200 MHz, CDCl_3) δ 8.63 (d, J = 5.7 Hz, 2H), 7.58 – 7.30 (m, 7H), 7.36 – 7.13 (m, 7H), 6.59 (s, 1H), 4.84 – 4.68 (m, 2H), 3.89 (s, 3H), 3.35 – 3.07 (m, 2H), 1.27 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 174.42, 173.04, 161.51, 161.12, 149.40, 148.81, 138.04, 136.11, 129.86, 129.09, 127.45, 126.03, 104.55, 55.73, 54.08, 38.35, 37.93, 37.75, 32.45, 30.45. ESI-MS (+) Calc. for $[\text{C}_{26}\text{H}_{32}\text{N}_6\text{O}_3]$ 476.57, found: 477.3 $[\text{M}+\text{H}]^+$.

N-((*S*)-1-(((2*S*,3*R*)-1-Amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**62**)

Yield 65%. Yellowish solid. $R_f = 0.3$ (ethyl acetate). Mp. 109 – 110 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.30 – 7.08 (m, 9H), 6.40 (d, $J = 14.4$ Hz, 1H), 6.26 (s, 1H), 5.00 – 4.76 (m, 1H), 4.52 (s, 2H), 4.17 – 4.06 (m, 2H), 3.98 (s, 3H), 3.20 – 3.09 (m, 2H), 1.27 (s, 9H), 1.11 (d, $J = 6.2$ Hz, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 171.79, 171.19, 160.58, 160.20, 137.80, 136.25, 134.62, 129.26, 128.78, 128.44, 127.84, 127.78, 127.21, 103.32, 74.00, 71.58, 60.39, 56.64, 38.86, 38.61, 31.91, 30.49, 21.02. ESI-MS (+) Calc. for $[\text{C}_{29}\text{H}_{37}\text{N}_5\text{O}_4]$ 519.64, found: 549.4 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*R*,3*S*)-1-Amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**63**)

Yield 61%. Yellowish solid. $R_f = 0.3$ (ethyl acetate). Mp. 119 – 120 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.27 – 7.04 (m, 9H), 6.37 (d, $J = 14.4$ Hz, 1H), 6.22 (s, 1H), 4.97 – 4.73 (m, 1H), 4.48 (s, 2H), 4.14 – 4.03 (m, 2H), 3.95 (s, 3H), 3.17 – 3.06 (dd, $J = 11.3, 5.1$ Hz, 2H), 1.23 (s, 9H), 1.08 (d, $J = 6.2$ Hz, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 171.79, 171.19, 160.58, 160.20, 137.80, 136.25, 134.62, 129.26, 128.78, 128.44, 127.84, 127.78, 127.21, 103.32, 74.00, 71.58, 60.39, 56.64, 38.86, 38.61, 31.91, 30.49, 21.02. ESI-MS (+) Calc. for $[\text{C}_{29}\text{H}_{37}\text{N}_5\text{O}_4]$ 519.64, found: 549.4 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*S*,3*R*)-1-Amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**64**)

Yield 73%. Yellowish solid. R_f = 0.3 (ethyl acetate). Mp. 177 – 178 °C. ^1H NMR (200 MHz, CDCl_3) δ . 7.27 – 7.20 (m, 4H), 7.14 (d, J = 8.5 Hz, 1H), 6.42 (s, 1H), 4.85 – 4.82 (m, 1H), 4.62 – 4.52 (s, 2H), 3.96 (s, 3H), 3.87 – 3.84 (m, 1H), 1.69 – 1.59 (m, 3H), 1.22 (s, 9H). 1.18 (d, J = 6.3 Hz, 3H), 0.90 (d, J = 6.0 Hz, 3H), 0.97 (d, J = 6.0 Hz, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 172.35, 171.66, 160.37, 160.31, 137.82, 134.69, 128.57, 128.00, 127.74, 103.21, 74.07, 71.71, 56.24, 52.12, 39.03, 38.69, 32.02, 30.59, 25.00, 23.01, 22.0. ESI-MS (+) Calc. for $[\text{C}_{26}\text{H}_{39}\text{N}_5\text{O}_4]$ 485.62, found: 508.5 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*R*,3*S*)-1-Amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**65**)

Yield 70%. Yellowish solid. R_f = 0.3 (ethyl acetate). Mp. 170 – 171 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.33 – 7.21 (m, 4H), 7.15 (d, J = 8.5 Hz, 1H), 6.34 (s, 1H), 4.85-4.82 (m, 1H), 4.64 – 4.53 (s, 2H), 3.98 (s, 3H), 3.88 – 3.86 (m, 1H), 1.71 – 1.60 (m, 3H), 1.24 (s, 9H), 1.21 (d, J = 6.3 Hz, 3H), 0.91 (d, J = 6.0 Hz, 3H), 0.89 (d, J = 6.0 Hz, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 171.72, 171.02, 159.73, 159.68, 137.18, 134.05, 127.94, 127.36, 127.10, 102.58, 73.43, 71.08, 55.60, 51.49, 38.39, 38.06, 31.39, 29.95, 24.37, 22.38, 21.44. ESI-MS (+) Calc. for $[\text{C}_{26}\text{H}_{39}\text{N}_5\text{O}_4]$ 485.62, found: 508.5 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*S*,3*R*)-1-Amino-3-(benzyloxy)-1-oxobutan-2-yl)amino)-3-(3-chlorophenyl)-1-oxopropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**66**)

Yield 65%. Yellowish solid. R_f = 0.2 (ethyl acetate). Mp. 102 – 104 °C. ^1H NMR (200 MHz, CD_3OD) δ 8.59 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 8.4 Hz, 1H), 7.26 (dd, J = 5.6, 2.2 Hz, 9H), 6.59 (s, 1H), 4.64 – 4.45 (m, 3H), 4.16 – 4.11 (m, 1H), 3.86 (s, 3H), 3.27 – 3.25 (m, 1H), 3.03 (dd, J = 14.7, 10.8 Hz, 1H), 1.28 (s, 9H), 1.22 (d, J = 6.3 Hz, 3H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.44, 173.31, 162.05, 161.35, 140.90, 139.31, 136.28, 134.96, 130.78, 130.33, 129.08, 128.62, 128.52, 128.44, 127.71, 104.84, 75.78, 72.24, 58.45, 55.77, 38.46, 37.34, 32.66, 30.66, 16.58. ESI-MS (+) Calc. for $[\text{C}_{29}\text{H}_{36}\text{ClN}_5\text{O}_4]$ 554.08, found: 577.1 $[\text{M}+\text{Na}]^+$.

Compounds **67** – **77** were synthesized by dehydration of the corresponding primary amide precursor **56** – **66** with cyanuric chloride (general procedure for dehydration of primary amide to nitrile, method A).

3-(*tert*-Butyl)-*N*-((*S*)-1-(((*S*)-1-cyano-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**67**)

Yield 89%. Yellowish solid. R_f = 0.6 (ethyl acetate: *n*-hexane; 6:4). Mp. 89 – 90 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.32 – 7.21 (m, 8H), 7.16 (dd, J = 6.4, 2.8 Hz, 2H), 6.90 (d, J = 8.2 Hz, 1H), 6.82 (d, J = 8.2 Hz, 1H), 6.35 (s, 1H), 5.04 – 5.02 (m, 1H), 4.82 – 4.80 (m, 1H), 4.02 (s, 3H), 3.21 – 3.07 (m, 2H), 3.00 – 2.98 (m, 2H), 1.33 – 1.30 (m, 9H).

^{13}C NMR (100 MHz, CDCl_3) δ 170.30, 160.25, 160.30, 135.55, 134.04, 133.37, 129.08, 129.04, 128.75, 128.72, 127.71, 127.26, 117.25, 103.06, 54.17, 41.48, 38.77, 38.35, 37.95, 31.75, 30.26. FT-IR (KBr, cm^{-1}) 3300.81, 2951.21, 2909.60, 2243.70, 1677.69, 1648.55, 1544.51, 1278.15, 1232.37, 986.82, 751.92, 670.52, 524.68, 424.97. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{32}\text{N}_5\text{O}_2]^+$ 458.25560, found: 458.2586 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 9.07. Purity 99.0%.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((*R*)-1-cyano-2-phenylethyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**68**)

Yield 92%. Yellowish solid. R_f = 0.6 (ethyl acetate: *n*-hexane; 6:4). Mp. 98 – 99 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.34 – 7.12 (m, 9H), 6.91 (d, J = 7.9 Hz, 1H), 6.34 (s, 1H), 5.09 – 5.05 (m, 1H), 4.94 – 4.91 (m, 1H), 4.00 (s, 3H), 3.12 (dd, J = 13.8, 6.4 Hz, 2H), 2.98 (dd, J = 13.8, 6.4 Hz, 2H), 1.30 (s, 9H). ^{13}C NMR (50 MHz, CDCl_3) δ 170.75, 160.43, 160.33, 135.98, 134.32, 133.78, 129.42, 129.33, 129.01, 128.86, 127.99, 127.42, 118.87, 103.48, 54.38, 41.82, 38.96, 38.48, 38.26, 31.98, 30.51. FT-IR (KBr, cm^{-1}) 3350.28, 3256.30, 2953.92, 2165.27, 1638.14, 1511.6, 1307.15, 1160.05, 1086.49, 906.70, 820.89, 694.21, 669.69, 543.02. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{32}\text{N}_5\text{O}_2]^+$ 458.25560, found: 458.2586 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 17.32. Purity 99.3%.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((*S*)-1-cyano-3-methylbutyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**69**)

Yield 84%. Yellowish solid. R_f = 0.7 (ethyl acetate: *n*-hexane; 6:4). Mp 81 – 82 °C. ^1H NMR (200 MHz, CD_3OD) δ 7.29 – 7.20 (m, 5H), 6.64 (s, 1H), 4.80 – 4.69 (m, 2H), 3.90

(s, 3H), 3.23 – 2.94 (m, 2H), 1.78 – 1.62 (m, 3H), 1.27 (s, 9H), 0.95 – 0.91 (m, 6H). ^{13}C NMR (50 MHz, CD_3OD) δ 173.33, 161.87, 161.51, 138.07, 136.59, 130.36, 129.56, 127.93, 119.68, 105.02, 56.04, 49.00, 42.00, 40.04, 38.69, 32.86, 30.88, 25.80, 22.35, 22.19. FT-IR (KBr, cm^{-1}) 3403.41, 3309.42, 3203.18, 2953.92, 2937.57, 1695.35, 1634.05, 1523.72, 1368.44, 1274.46, 1245.86, 1168.22, 835.58, 702.38, 669.69, 567.54. HRMS (+) Calc. for $[\text{C}_{24}\text{H}_{34}\text{N}_5\text{O}_2]^+$ 423.27125, found: 424.27572 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_{R} (min) = 9.16. Purity 97.4%.

3-(tert-Butyl)-N-((S)-1-(((R)-1-cyano-3-methylbutyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1H-pyrazole-5-carboxamide (70)

Yield 79%. White solid. R_f = 0.7 (ethyl Acetate: *n*-hexane; 6:4). Mp. 91 – 94 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.32 – 7.21 (m, 3H), 6.98 (d, J = 8.2 Hz, 1H), 6.88 (d, J = 8.2 Hz, 1H), 6.34 (s, 1H), 4.92 – 4.86 (m, 1H), 4.77 – 4.71 (m, 1H), 3.99 (s, 3H), 3.29 – 3.19 (m, 2H), 1.63 – 1.46 (m, 3H), 1.27 (s, 9H), 0.88 (2d, J = 5.9 Hz, 6H). ^{13}C NMR (50 MHz, CDCl_3) δ 171.28, 161.15, 161.04, 136.68, 135.04, 129.97, 129.56, 128.05, 119.17, 104.16, 55.16, 41.95, 39.62, 39.12, 32.67, 31.19, 25.31, 22.79, 22.53. FT-IR (KBr, cm^{-1}) 3350.28, 3256.30, 2953.92, 2165.27, 1638.14, 1511.46, 1307.15, 1160.05, 1086.49, 906.70, 820.89, 694.21, 669.69, 543.02. HRMS (+) Calc. for $[\text{C}_{24}\text{H}_{34}\text{N}_5\text{O}_2]^+$ 423.27125, found: 424.27572 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_{R} (min) = 9.42. Purity 99.1%.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((*S*)-2-(3-chlorophenyl)-1-cyanoethyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**71**)

Yield 50%. Yellowish solid. R_f = 0.6 (ethyl acetate: *n*-hexane; 6:4). Mp 132 – 133 °C. ^1H NMR (200 MHz, $\text{CD}_3\text{OD}/\text{CDCl}_3$) δ 7.54 – 7.35 (m, 9H), 6.75 (s, 1H), 5.19 – 5.11 (m, 1H), 4.51 – 4.48 (m, 1H), 4.17 (s, 3H), 3.33 – 3.21 (m, 4H), 1.49 (s, 9H). ^{13}C NMR (50 MHz, CD_3OD) δ 172.23, 161.25, 161.03, 137.40, 136.96, 135.83, 135.15, 130.68, 130.07, 129.87, 129.23, 128.42, 128.30, 127.69, 118.18, 104.55, 55.12, 42.41, 38.76, 38.62, 32.51, 30.78. FT-IR (cm^{-1}) 3288.99, 2925.31, 2855.85, 2161.18, 1666.74, 1634.05, 1544.15, 1507.38, 1450.17, 1266.29, 1221.34, 1074.23, 861.75, 747.33, 706.47, 694.21. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{31}\text{ClN}_5\text{O}_2]^+$ 491.21663, found: 492.21034 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 9.61. Purity > 99.9 %.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((*S*)-1-cyano-2-(pyridin-4-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**72**)

Yield 35%. Yellowish wax. R = 0.4 (ethyl acetate: *n*-hexane; 6:4). ^1H NMR (400 MHz, CD_3OD) δ 8.44 (s br, 2H), 7.38 (d, J = 5.3 Hz, 2H), 7.30 – 7.22 (m, 5H), 6.63 (s, 1H), 5.14 (t, J = 7.5 Hz, 1H), 4.66 (dd, J = 8.6, 6.8 Hz, 1H), 3.94 (s, 3H), 3.26 – 3.13 (m, 4H), 1.30 (s, 9H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 171.17, 170.90, 159.29, 158.46, 137.89, 136.81, 135.23, 134.81, 129.27, 128.86, 128.13, 127.92, 126.93, 126.34, 118.94, 60.46, 53.87, 53.24, 41.15, 31.39, 30.18, 13.74. FT-IR (cm^{-1}) 3264.47, 2953.92, 2913.05, 2851.76, 2161.43, 1662.66, 1605.45, 1548.24, 1466.62, 1204.99, 1115.10, 996.59, 800.45, 735.07, 681.95. HRMS (+) Calc. for $[\text{C}_{26}\text{H}_{31}\text{N}_6\text{O}_2]^+$ 459.25085, found: 459.25627 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 6.84. Purity 98.6%.

N-((*S*)-1-(((1*R*,2*R*)-2-(Benzyloxy)-1-cyanopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**73**)

Yield 80%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 6:4). Mp. 115 – 116 °C. ^1H -NMR (200 MHz, CDCl_3) δ 7.35 – 7.26 (m, 10H), 6.95 (d, J = 8.7 Hz, 1H), 6.61 (d, J = 7.0 Hz, 1H), 6.30 (s, 1H), 4.88 – 4.85 (m, 2H), 4.62 – 4.58 (m, 1H), 4.03 (s, 3H), 3.82 – 3.78 (m, 1H), 3.29 – 3.24 (m, 2H), 1.31 (s, 9H), 1.03 (d, J = 6.0 Hz, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 170.30, 159.83, 159.62, 136.42, 135.27, 133.75, 128.67, 128.41, 128.00, 127.60, 127.30, 126.87, 116.63, 102.65, 72.60, 70.91, 53.85, 44.51, 38.33, 37.60, 31.40, 29.94, 15.29. FT-IR (cm^{-1}) 3354.37, 3252.21, 2181.61, 1654.61, 1654.48, 1540.07, 1486.95, 1290.81, 1151.87, 1086.48, 894.44, 808.63, 710.56, 661.32, 543.02. HRMS (+) Calc. for $[\text{C}_{29}\text{H}_{36}\text{N}_5\text{O}_3]^+$ 502.28182, found: 502.28095 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 9.54. Purity 99.4%.

N-((*S*)-1-(((1*S*,2*S*)-2-(Benzyloxy)-1-cyanopropyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**74**).

Yield 82%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 6:4). Mp. 123 – 124 °C. ^1H NMR (200 MHz, CDCl_3) δ 7.36 – 7.25 (m, 10H), 6.98 (d, J = 8.7 Hz, 1H), 6.65 (d, J = 7.0 Hz, 1H), 6.32 (s, 1H), 4.91 – 4.83 (m, 2H), 4.63 – 4.60 (m, 1H), 4.05 (s, 3H), 3.80 (s br, 1H), 3.24 – 3.20 (m, 2H), 1.33 (s, 9H), 1.05 (d, J = 6.0 Hz, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 171.01, 160.54, 160.34, 137.14, 135.98, 134.46, 129.39, 129.12, 128.71, 128.32, 128.02, 127.59, 117.34, 103.37, 72.85, 71.15, 54.56, 45.22, 39.04, 38.32, 32.12, 30.65, 16.00. FT-IR (cm^{-1}) 2376.73, 2958.00, 2169.35, 1642.22, 15400.07, 1499.21, 1442.00, 1290.81, 1225.43, 1111.01, 1033.37, 988.42, 743.25,

690.12. HRMS (+) Calc. for $[C_{29}H_{36}N_5O_3]^+$ 502.28182, found: 502.28095 $[M+H]^+$. HPLC (protocol B): t_R (min) = 9.43. Purity 98.3%.

N-((*S*)-1-(((1*R*,2*R*)-2-(Benzyloxy)-1-cyanopropyl)amino)-4-methyl-1-oxopentan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**75**)

Yield 78%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 6:4). Mp. 96 – 97 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.93 (d, J = 8.1 Hz, 1H), 8.43 (d, J = 7.6 Hz, 1H), 7.38 – 7.29 (m, 5H), 6.88 (s, 1H), 5.07 – 5.04 (m, 1H), 4.64 – 4.61 (m, 2H), 4.59 – 4.56 (m, 1H), 3.97 – 3.94 (m, 3H), 3.86 – 3.84 (m, 1H), 1.72 – 1.64 (m, 2H), 1.50 – 1.46 (m, 2H), 1.26 – 1.20 (m, 12H), 0.93 (d, J = 6.3 Hz, 3H), 0.88 (d, J = 6.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.36, 159.56, 158.66, 137.92, 134.92, 128.10, 127.53, 127.45, 117.82, 103.73, 73.39, 70.40, 51.05, 44.75, 38.44, 31.53, 30.31, 24.25, 22.91, 21.19, 15.69. FT-IR (cm^{-1}) 3378.89, 3350.28, 3186.83, 2958.00, 2116.43, 1674.91, 1650.40, 1531.90, 1368.44, 1262.20, 1160.05, 1057.89, 1033.37, 780.02, 735.07, 649.26, 604.31. HRMS (+) Calc. for $[C_{26}H_{38}N_5O_3]$ 468.29746, found: 468.29785 $[M+H]^+$. HPLC (protocol B): t_R (min) = 9.61. Purity 98.3%.

N-((*S*)-1-(((1*S*,2*S*)-2-(Benzyloxy)-1-cyanopropyl)amino)-4-methyl-1-oxopentan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**76**)

Yield 70%. White solid. R_f = 0.5 (ethyl acetate: *n*-hexane; 6:4). Mp. 110 – 111 °C. 1H NMR (400 MHz, $CDCl_3$) δ 7.27 – 7.20 (m, 5H), 7.14 (d, J = 8.5 Hz, 1H), 6.42 (d, J = 7.8 Hz, 1H), 6.33 (s, 1H), 4.85 – 4.82 (m, 1H), 4.62 – 4.52 (m, 3H), 3.96 (s, 3H), 3.87 – 3.84 (m, 1H), 1.69 – 1.59 (m, 3H), 1.22 (s, 9H), 1.18 (d, J = 6.3 Hz, 3H), 1.24 – 1.19 (m, 12H), 0.92 – 0.88 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.10, 160.65, 160.51,

137.21, 134.43, 128.66, 128.23, 127.92, 117.45, 103.16, 73.69, 71.80, 51.75, 45.28, 40.81, 38.06, 32.10, 30.62, 25.04, 22.97, 22.13, 16.36. FT-IR (cm⁻¹) 3333.94, 3284.90, 2962.09, 2868.10, 2255.16, 1647.44, 1540.07, 1507.38, 1442.00, 1274.46, 1245.86, 988.42, 739.16, 661.52. HRMS (+) Calc. for [C₂₆H₃₈N₅O₃] 468.29746, found: 468.29785 [M+H]⁺. HPLC (protocol B): t_R (min) = 9.56. Purity 96.9%.

N-((*S*)-1-(((1*R*,2*R*)-2-(Benzyloxy)-1-cyanopropyl)amino)-3-(3-chlorophenyl)-1-oxopropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**77**)

Yield 50%. Yellowish wax. *R*_f = 0.4 (ethyl acetate: *n*-hexane; 6:4). ¹H NMR (400 MHz, CDCl₃) δ 7.30 – 7.13 (m, 6H), 7.04 (dt, *J* = 7.0, 1.7 Hz, 1H), 6.62 (dd, *J* = 17.8, 8.2 Hz, 2H), 6.25 (s, 1H), 4.77 – 4.71 (m, 2H), 4.48 (dd, *J* = 36.1, 11.7 Hz, 2H), 3.95 (s, 3H), 3.82 – 3.74 (m, 1H), 3.11 – 3.00 (m, 2H), 1.20 (s, 9H), 1.08 (d, *J* = 6.3 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 170.73, 160.54, 160.12, 137.80, 136.91, 134.84, 134.30, 130.44, 129.51, 128.71, 128.34, 128.03, 127.84, 127.62, 117.06, 103.35, 73.35, 71.72, 54.28, 45.07, 39.06, 38.13, 32.07, 30.58, 16.21. FT-IR (cm⁻¹) 3357.24, 3264.21, 2193.15, 1654.33, 1538.29, 1487.88, 1209.18, 1141.78, 1036.43, 863.33, 807.36, 701.44, 658.23, 534.57. ESI-MS (+) Calc. for [C₂₉H₃₄ClN₅O₃] 536.24284, found: 536.24235 [M+H]⁺. HPLC (protocol B): t_R (min) = 12.06. Purity 96.5%.

Compounds **78** – **81** were synthesized from compounds **62** – **65** by removal of the benzoyl group through hydrogenation on Pd/C (general procedure for removal of the benzyl protecting group, method A).

N-((*S*)-1-(((2*S*,3*R*)-1-Amino-3-hydroxy-1-oxobutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide. (**78**)

Yield 92%. Colorless wax. R_f = 0.2 (ethyl acetate). ^1H NMR (200 MHz, CDCl_3) δ 8.03 (s, 1H), 7.36 – 6.94 (m, 5H), 6.54 (s, 1H), 4.44 – 4.39 (m, 2H), 4.03 – 4.01 (m, 1H), 3.96 (s, 3H), 3.18 – 3.14 (m, 1H), 2.87 (s, 1H), 1.30 (s, 9H), 1.03 – 1.01 (m, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 173.54, 172.15, 161.25, 160.35, 136.25, 135.98, 134.58, 129.36, 128.76, 127.25, 66.60, 58.51, 55.55, 54.30, 38.70, 31.98, 30.51, 18.92. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{31}\text{N}_5\text{O}_4]$ 429.51, found: 452.4 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*R*,3*S*)-1-Amino-3-hydroxy-1-oxobutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide. (**79**)

Yield 95%. Colorless wax. R_f = 0.2 (ethyl acetate). ^1H NMR (200 MHz, CDCl_3) δ 8.01 (s, 1H), 7.36 – 6.98 (m, 5H), 6.59 (s, 1H), 4.42 – 4.38 (m, 2H), 4.04 – 4.01 (m, 1H), 3.96 (s, 3H), 3.12 – 3.09 (m, 1H), 2.89 (s, 1H), 1.33 (s, 9H), 1.03 – 0.99 (m, 3H). ^{13}C NMR (50 MHz, CDCl_3) δ 173.54, 172.15, 161.25, 160.35, 136.25, 135.98, 134.58, 129.36, 128.76, 127.25, 66.60, 58.51, 55.55, 54.30, 38.70, 31.98, 30.51, 18.92. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{31}\text{N}_5\text{O}_4]$ 429.51, found: 452.4 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*S*,3*R*)-1-Amino-3-hydroxy-1-oxobutan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**80**)

Yield 90%. Colorless wax. R_f = 0.2 (ethyl acetate). ^1H NMR (200 MHz, CDCl_3) δ 7.37 (d, J = 3.9 Hz, 1H), 6.74 – 6.71 (m, 2H), 6.42 (s, 1H), 4.71 – 4.68 (m, 1H), 4.42 – 4.38 (m, 2H), 4.06 (s, 3H), 1.71 – 1.68 (m, 3H), 1.27 (s, 9H), 1.14 – 1.11 (m, 3H), 0.98 – 0.92 (m, 6H). ESI-MS (+) Calc. for $[\text{C}_{19}\text{H}_{33}\text{N}_5\text{O}_4]$ 395.51, found: 418.5 $[\text{M}+\text{Na}]^+$.

N-((*S*)-1-(((2*R*,3*S*)-1-Amino-3-hydroxy-1-oxobutan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-3-(*tert*-butyl)-1-methyl-1*H*-pyrazole-5-carboxamide (**81**)

Yield 93%. Colorless wax. R_f = 0.2 (ethyl acetate). ^1H NMR (200 MHz, CDCl_3) δ 7.34 (d, J = 3.9 Hz, 1H), 6.79 – 6.75 (m, 2H), 6.46 (s, 1H), 4.73 – 4.70 (m, 1H), 4.45 – 4.41 (m, 2H), 4.11 (s, 3H), 1.77 – 1.74 (m, 3H), 1.28 (s, 9H), 1.19 – 1.17 (m, 3H), 0.99 – 0.91 (m, 6H). ESI-MS (+) Calc. for $[\text{C}_{19}\text{H}_{33}\text{N}_5\text{O}_4]$ 395.51, found: 418.5 $[\text{M}+\text{Na}]^+$.

Compounds **82** – **85** have been synthesized by dehydration of the corresponding primary amide precursor **78** – **81** with trifluoroacetic anhydride (general procedure for dehydration of primary amide to nitrile, method B).

3-(*tert*-Butyl)-*N*-((*S*)-1-(((1*R*,2*R*)-1-cyano-2-hydroxypropyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**82**)

Yield 45%. White solid. $R_f = 0.7$ (ethyl acetate). Mp. 159 – 160 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.32 – 7.26 (m, 4H), 7.26 – 7.21 (m, 1H), 6.65 (s, 1H), 4.84 – 4.79 (m, 2H), 3.96 (s, 3H), 3.88 – 3.84 (m, 1H), 3.20 (dd, $J = 13.6, 7.1$ Hz, 1H), 3.10 (dd, $J = 13.6, 7.1$ Hz, 1H), 1.31 (s, 9H), 1.07 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.35, 162.06, 161.60, 138.15, 136.69, 130.42, 129.57, 127.98, 118.24, 105.04, 67.67, 56.05, 38.86, 38.68, 32.90, 30.87, 18.89, 18.55. FT-IR (KBr, cm^{-1}) 3293.08, 2953.92, 2913.05, 2868.10, 1646.10, 1540.07, 1446.08, 1286.72, 1237.68, 1098.75, 922.51, 739.16, 689.30, 469.47. HRMS (+) Calc. for $[\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_3]^+$ 412.23486, found: 412.23811 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 7.65. Purity 97.8%.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((1*S*,2*S*)-1-cyano-2-hydroxypropyl)amino)-1-oxo-3-phenylpropan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**83**)

Yield 42%. White solid. $R_f = 0.7$ (ethyl acetate). Mp. 145 – 147 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.31 – 7.23 (m, 5H), 7.07 (d, $J = 8.0$ Hz, 1H), 6.67 (d, $J = 7.5$ Hz, 1H), 6.31 (s, 1H), 4.85 – 4.84 (m, 1H), 4.72 – 4.68 (m, 1H), 4.15 – 4.10 (m, 1H), 4.02 (s, 3H), 3.21 – 3.15 (m, 2H), 1.29 – 1.27 (m, 9H), 1.08 (d, $J = 6.2$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.41, 171.26, 160.58, 135.89, 134.27, 129.38, 129.14, 127.65, 117.25, 103.52, 67.39, 54.76, 47.02, 39.11, 38.38, 32.09, 30.59, 19.02. FT-IR (KBr, cm^{-1}) 3289.11, 2955.43, 2918.55, 2860.11, 1649.54, 1543.16, 1444.02, 1277.62, 1233.66, 1091.87, 944.77, 745.55, 690.34, 477.11. HRMS (+) Calc. for $[\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_3]^+$ 412.23486, found: 412.23811 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 7.71. Purity 95.7%.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((1*R*,2*R*)-1-cyano-2-hydroxypropyl)amino)-4-methyl-1-oxopentan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**84**)

Yield 33%. White solid. R_f = 0.5 (ethyl acetate). Mp. 118 – 119 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.56 – 7.51 (m, 1H), 6.80 – 6.75 (m, 1H), 6.43 (s, 1H), 4.87 – 4.83 (m, 1H), 4.65 – 4.63 (m, 1H), 4.15 – 4.11 (m, 1H), 4.06 (s, 3H), 1.31 – 1.20 (m, 15H), 0.97 (2d, J = 6.2 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.32, 161.62, 162.14, 135.77, 117.22, 104.56, 67.34, 52.68, 41.12, 38.28, 31.46, 32.15, 26.11, 23.56, 21.65, 19.03. FT-IR (KBr, cm^{-1}) 3297.16, 2953.92, 2913.05, 2868.10, 1646.31, 1535.89, 1503.29, 1462.52, 1364.36, 1282.63, 1172.38, 1131.44, 996.59, 730.99, 592.05. HRMS (+) Calc. for $[\text{C}_{19}\text{H}_{32}\text{N}_5\text{O}_3]^+$ 378.25071, found: 378.25231 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 8.08. Purity 96.7%.

3-(*tert*-Butyl)-*N*-((*S*)-1-(((1*S*,2*S*)-1-cyano-2-hydroxypropyl)amino)-4-methyl-1-oxopentan-2-yl)-1-methyl-1*H*-pyrazole-5-carboxamide (**85**)

Yield 35%. White solid. R_f = 0.5 (ethyl acetate). Mp. 111 – 113 °C. ^1H NMR (400 MHz, CD_3OD) δ 6.78 (s, 1H), 4.88 – 4.84 (m, 1H), 4.65 – 4.60 (m, 1H), 4.07 – 4.05 (m, 1H), 4.05 (s, 3H), 1.81 – 1.61 (m, 4H), 1.32 (s, 9H), 1.26 (d, J = 6.3 Hz, 3H), 1.00 (2d, J = 6.3 Hz, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 175.01, 162.46, 161.76, 136.76, 118.59, 105.26, 68.00, 53.28, 41.76, 39.00, 33.07, 31.04, 26.27, 23.54, 21.94, 19.44. FT-IR (KBr, cm^{-1}) 3293.08, 2962.09, 2917.14, 2847.67, 1646.31, 1540.07, 1458.34, 1278.55, 1131.44, 1078.32, 1000.68, 853.58, 812.71, 780.02, 730.99, 559.36. HRMS (+) Calc. for $[\text{C}_{19}\text{H}_{32}\text{N}_5\text{O}_3]^+$ 378.25071, found: 378.25231 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 7.94. Purity 98.6%.

Compound **86** was synthesized by removal of the benzyl group from compound **77** with DDQ (general procedure for removal of the benzyl protecting group, method B).

3-(tert-butyl)-N-((S)-3-(3-chlorophenyl)-1-(((1R,2R)-1-cyano-2-hydroxypropyl)amino)-1-oxopropan-2-yl)-1-methyl-1H-pyrazole-5-carboxamide (86)

Yield 32%. White solid. R_f = 0.3 (ethyl acetate). Mp. 102 – 103 °C. ^1H NMR (400 MHz, CD_3OD) δ 7.35 – 7.28 (m, 4H), 6.69 (s, 1H), 4.87 – 4.83 (m, 2H), 3.99 (s, 3H), 3.90 – 3.84 (m, 1H), 3.24 (dd, J = 13.6, 7.1 Hz, 1H), 3.08 (dd, J = 13.6, 6.5 Hz, 1H), 1.34 (s, 9H), 1.11 (d, J = 6.3 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 175.23, 163.23, 151.94, 151.48, 128.04, 126.57, 120.30, 120.10, 119.52, 119.45, 117.86, 108.12, 94.92, 57.55, 45.93, 28.74, 28.56, 22.78, 20.75, 18.78. FT-IR (KBr, cm^{-1}) 3284.90, 3056.07, 2958.00, 2925.31, 2868.10, 1638.14, 1556.41, 1499.21, 1462.43, 1364.36, 1270.37, 1229.51, 1098.75, 878.09, 730.99, 702.38, 453.12. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{29}\text{ClN}_5\text{O}_3]^+$ 446.19589, found: 446.19745 $[\text{M}+\text{H}]^+$. HPLC (protocol B): t_R (min) = 8.55. Purity 99.7%.

8.1.14. Synthesis and characterization of compounds presented in chapter IV.

Scheme 11. General synthesis of compounds **87 – 90** and **91 – 98**.

Reagents and conditions: (a) SOCl_2 , CH_3OH , reflux, 1 h $\rightarrow$ rt, overnight; (b) ArC(=O)CF_3 , K_2CO_3 , CH_3OH , 50 °C, 18 h; (c) 1M $\text{Zn}(\text{BH}_4)_2$ in DME, $\text{ACN}/\text{CH}_3\text{OH}$ (5:1), -40/-45 °C, 3 h; (d) NaBH_4 , THF, rt, 6 h. Source: Own author.

Scheme 12. Synthesis of compound **99**.

Reagents and conditions: a) Isobutyl chloroformate, NH_4Cl 2M, DIPEA, DMF, 0 °C to rt, 20 h; b) TFA, CH_2Cl_2 , 0 °C to rt, 2 h. Source: Own author.

Scheme 13. Synthesis of compounds **100 – 199**.

Reagents and conditions: (a) HATU, DIPEA, amine, rt, 22 h. Source: Own author.

Scheme 14. Synthesis of compounds **120** and **121**.

Reagents and conditions: suitable boronic acid pinacol ester, PdCl_2dppf , DMF, 80 °C, 3 h, argon atmosphere. Source: Own author.

Scheme 15. Synthesis of compounds **123** and **124**.

Reagents and conditions: (a) HATU, DIPEA, NH_2XCN , rt, 22 h; (b) formic acid, rt, 18 h; (c) HATU, DIPEA, benzoic acid, rt, 22h. Source: Own author.

Table 10. List of Nequimed codes of compounds described in chapter IV.

Scheme 13		Scheme 14		Scheme 15	
Number	Nequimed Code	Number	Nequimed Code	Number	Nequimed Code
100	Neq0672	120	Neq0665	123	Neq0818
101	Neq0635	121	Neq0820	124	Neq0819
102	Neq0641				
103	Neq0642				
104	Neq0594				
105	Neq0631				
106	Neq0636				
107	Neq0669				

Number	Nequimed Code		
108	Neq0668		
109	Neq0715		
110	Neq0716		
111	Neq0670		
112	Neq0643		
113	Neq0658		
114	Neq0659		
115	Neq0645		
116	Neq0671		
117	Neq0633		
118	Neq0629		
119	Neq0630		

Neq0587 was donated by AstraZeneca, Mölndal, Sweden

Compounds **87** – **90** were synthesized by the corresponding enantiomeric pure amino acids with SOCl_2 (general method for the synthesis of amino esters).

*1-methoxy-4-methyl-1-oxopentan-2-aminium chloride (**87**)*

Yield 87%. White solid. Mp. 149 – 151 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.86 (s, 2H), 3.87 (t, J = 7.0 Hz, 1H), 3.70 (s, 3H), 1.76 – 1.58 (m, 3H), 0.85 (d, J = 6.4 Hz, 6H). ESI-MS (+) Calc. for $[\text{C}_7\text{H}_{16}\text{NO}_2]$ 145.11, found: 146.25 $[\text{M}+\text{H}]^+$ (HUGHES et al., 2007).

*1-methoxy-1-oxo-3-phenylpropan-2-aminium chloride (**88**)*

Yield 88%. White solid. Mp. 156 – 160 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.86 (s, 2H), 7.36 – 7.23 (m, 5H), 4.19 – 1.16 (m, 1H), 3.63 (s, 3H), 3.24 (dd, J = 13.9, 5.5 Hz, 1H), 3.10 (dd, J = 14.2, 5.7 Hz, 1H). ESI-MS (+) Calc. for $[\text{C}_{10}\text{H}_{13}\text{NO}_2]$ 179.10, found: 180.05 $[\text{M}+\text{H}]^+$ (HUGHES et al., 2007).

1-methoxy-3-(4-methoxyphenyl)-1-oxopropan-2-aminium chloride (89)

Yield 90%. White solid. Mp. 157 – 159 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.66 (s, 2H), 7.14 – 7.10 (m, 2H), 6.87 – 6.83 (m, 2H), 4.19 – 1.16 (m, 1H), 3.70 (s, 3H), 3.63 (s, 3H), 3.10 (dd, J = 13.9, 5.5 Hz, 1H), 3.01 (dd, J = 14.2, 5.7 Hz, 1H). ESI-MS (+) Calc. for $[\text{C}_{11}\text{H}_{15}\text{NO}_3]$ 209.11, found: 210.1 $[\text{M}+\text{H}]^+$.

3-(1H-indol-3-yl)-1-methoxy-1-oxopropan-2-aminium chloride (90)

Yield 77%. White solid. Mp. 157 – 159 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.66 (s, 2H), 7.14 – 7.10 (m, 2H), 6.87 – 6.83 (m, 2H), 4.19 – 1.16 (m, 1H), 3.70 (s, 3H), 3.63 (s, 3H), 3.10 (dd, J = 13.9, 5.5 Hz, 1H), 3.01 (dd, J = 14.2, 5.7 Hz, 1H). ESI-MS (+) Calc. for $[\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_2]$ 218.25, found: 219.17 $[\text{M}+\text{H}]^+$.

Compounds **91** – **98** were synthesized from the corresponding **87** – **90** in two steps. First, it was formed the imine with the corresponding amino esters (**91** – **98**) and the selected trifluoroacetophenone (general procedure for imine synthesis). The imine was used without further purification, and it was reduced to the corresponding amine **87** – **90** (general procedure for imine reduction to amine). Characterization of compounds **91**–**98** is reported as a mixture of diastereoisomers. NMR data lists peaks for both isomers.

(2S)-4-methyl-2-((2,2,2-trifluoro-1-phenylethyl)amino)pentanoic acid (91)

Yield 32%. White solid. Mp. 89 – 94 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.44 – 7.31 (m, 5H), 4.35 (q, J = 7.8 Hz, 1H), 2.80 (dd, J = 9.3, 4.8 Hz, 1H), 1.87 – 1.69 (m, 1H), 1.47 – 1.40 (m, 1H), 1.32 – 1.25 (m, 1H), 0.79 (d, J = 6.7 Hz, 3H), 0.64 (d, J = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 176.0, 133.9, 129.6, 129.5, 128.9, 127.2, 124.4, 62.4, 62.1, 61.9, 56.2, 42.4, 24.5, 23.3, 21.9. ESI-MS (+) Calc. for $[\text{C}_{17}\text{H}_{18}\text{F}_3\text{NO}_2]$ 289.29, found: 290.33 $[\text{M}+\text{H}]^+$.

(2S)-2-((1-(4-bromophenyl)-2,2,2-trifluoroethyl)amino)-4-methylpentanoic acid (92)

61% Yield 61%. White solid. Mp. 85 – 90 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.67 – 7.58 (m, 1H), 7.44 – 7.40 (m, 2H), 4.43 (q, J = 7.6 Hz, 1H), 4.11 – 3.99 (m, 2H), 2.82 (dd, J = 9.2, 4.2 Hz, 1H), 1.86 – 1.71 (m, 1H), 1.67 – 1.56 (m, 2H), 1.49 – 1.43 (m, 1H), 1.36 – 1.29 (m, 1H), 0.82 (d, J = 6.7 Hz, 3H), 0.68 (d, J = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 176.4, 175.9, 170.8, 133.6, 131.9, 131.7, 131.6, 131.1, 128.8, 126.6, 124.4, 122.9, 63.8, 62.0, 61.7, 61.5, 61.3, 56.3, 42.3, 25.2, 24.5, 23.3, 21.9, 21.1. ESI-MS (+) Calc. for $[\text{C}_{14}\text{H}_{17}\text{BrF}_3\text{NO}_2]$ 368.19, found: 369.26 $[\text{M}+\text{H}]^+$.

(2S)-2-((1-([1,1'-biphenyl]-4-yl)-2,2,2-trifluoroethyl)amino)-4-methyl pentanoic acid (93)

56% Yield 56%. White solid. Mp. 143 – 145 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.72 – 7.67 (m, 6H), 7.59 – 7.46 (m, 6H), 7.40 – 7.36 (m, 2H), 4.48 – 4.39 (m, 2H), 1.91 – 1.78 (m, 2H), 1.52 – 1.34 (m, 4H), 0.90 (d, J = 6.6 Hz, 1H), 0.89 (d, J = 6.7, 1H), 0.84 (d, J = 6.7 Hz, 3H), 0.70 (d, J = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 176.5, 176.0, 141.4, 140.0, 139.9, 133.1, 130.1, 129.5, 129.4, 128.2, 128.1, 127.2, 127.1, 62.0 (m, 2C), 59.0, 56.3, 42.4, 42.3, 24.7, 24.5, 23.4, 23.3, 22.2, 22.0. ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{22}\text{F}_3\text{NO}_2]$ 365.39, found: 366.16 $[\text{M}+\text{H}]^+$.

(2S)-3-phenyl-2-((2,2,2-trifluoro-1-phenylethyl)amino)propanoic acid (94)

Yield 67%. White solid. Mp. 160 – 163 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.41 – 7.12 (m, 10H), 4.41 (q, J = 7.7 Hz, 1H), 3.09 – 3.06 (m, 1H), 2.89 (dd, J = 13.4, 5.4 Hz, 1H), 2.81 (dd, J = 13.4, 8.1 Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.8, 174.7, 138.3, 138.2, 133.7, 129.8, 129.4, 129.3, 128.8, 128.4, 126.9, 126.8, 124.6, 122.4, 62.1 (q, J = 27.9 Hz), 59.6, 39.0. ESI-MS (+) Calc. for $[\text{C}_{17}\text{H}_{16}\text{F}_3\text{NO}_2]$ 323.31, found: 324.26 $[\text{M}+\text{H}]^+$.

(S)-2-(((*R*)-1-(4-bromophenyl)-2,2,2-trifluoroethyl)amino)-3-phenyl propanoic acid
(**95**)

Yield 59%. White solid. Mp. 98 – 102 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.41 – 7.14 (m, 9H), 4.46 (q, J = 7.6 Hz, 1H), 3.13 – 3.19 (m, 1H), 2.97 – 2.91 (m, 1H), 2.86 – 2.81 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.7, 138.3, 133.7, 132.4, 131.9, 130.8, 129.8, 129.3, 128.8, 128.4, 126.7, 122.1, 62.15 (q, J = 27.9 Hz), 59.6, 39.1. ESI-MS (+) Calc. for $[\text{C}_{17}\text{H}_{15}\text{BrF}_3\text{NO}_2]$ 402.20, found: 403.11 $[\text{M}+\text{H}]^+$.

(2S)-2-((1-(3-chlorophenyl)-2,2,2-trifluoroethyl)amino)-3-phenylpropanoic acid (**96**)

Yield 71%. White solid. Mp. 74 – 77 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.38 – 7.13 (m, 8H), 7.04 (s, 1H), 6.93 (d, J = 7.7 Hz, 1H), 4.18 (q, J = 7.0 Hz, 1H), 3.35 (m, 1H), 3.04 (dd, J = 13.8, 7.1 Hz, 1H), 2.86 (dd, J = 13.7, 9.2 Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 178.5, 178.2, 136.1, 136.0, 135.8, 134.7, 134.7, 134.5, 130.1, 129.9, 129.6, 129.5, 129.3, 129.2, 128.8, 128.7, 127.4, 127.3, 127.0, 126.6, 126.0, 125.6, 123.8, 123.3, 62.3-63.1 (m, 2C), 59.2, 39.2, 39.0. ESI-MS (+) Calc. for $[\text{C}_{17}\text{H}_{15}\text{ClF}_3\text{NO}_2]$ 357.08, found: 358.14 $[\text{M}+\text{H}]^+$.

(S)-3-(4-methoxyphenyl)-2-(((*R*)-2,2,2-trifluoro-1-phenylethyl)amino) propanoic acid (**97**)

Yield 56%. White solid. Mp. 157 – 159 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.39 – 7.19 (m, 6H), 7.03 – 7.00 (m, 2H), 6.81 – 6.77 (m, 2H), 4.38 (q, $J = 7.7$ Hz, 1H), 3.69 (s, 3H), 3.03 (dd, $J = 7.5, 5.8$ Hz, 1H), 2.81 (dd, $J = 13.6, 5.6$ Hz, 1H), 2.74 (dd, $J = 13.6, 7.7$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.7, 158.3, 133.7, 130.7, 129.9, 129.4, 129.3, 128.8, 127.1, 124.3, 113.9, 62.5, 62.2, 61.9, 61.7, 59.7, 55.4, 38.1. ESI-MS (+) Calc. for $[\text{C}_{18}\text{H}_{18}\text{F}_3\text{NO}_2]$ 353.33, found: 354.41 $[\text{M}+\text{H}]^+$.

(2S)-3-(1*H*-indol-3-yl)-2-((2,2,2-trifluoro-1-phenylethyl)amino)propanoic acid (**98**)

Yield 56%. White solid. Mp. 85 – 87 °C. ^1H NMR (400 MHz, CD_3OD) δ 7.30 (dt, $J = 7.8, 1.0$, 1H), 7.26 (dt, $J = 8.0, 0.7$ Hz, 1H), 7.15 – 7.13 (m, 1H), 7.00 (m, 3H), 6.90 (d, $J = 8.0$ Hz, 1H), 6.82 (m, 1H), 4.18 (q, $J = 7.6$ Hz, 1H), 3.19 (d, $J = 4.9$ Hz, 1H), 3.12 (m, 1H), 2.91 (dd, $J = 14.2, 8.3$ Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ 175.1, 136.4, 132.0, 129.1, 128.5, 128.0, 123.2, 122.5, 119.9, 118.5, 112.2, 109.8, 91.7, 63.6, 59.0, 28.8. ESI-MS (+) Calc. for $[\text{C}_{19}\text{H}_{17}\text{F}_3\text{N}_2\text{O}_2]$ 332.34, found: 363.44 $[\text{M}+\text{H}]^+$.

Synthesis of compound **99** was performed in two steps. First, it was synthesized compound **34**, as described above, and then the primary amide was dehydrated to the desired nitrile (general procedure for dehydration of primary amide to nitrile,

method B). To confirm the absence of racemization the same synthesis was performed with Boc-D-phenylalanine and then analyzed by HPLC (see appendix B).

(S)-tert-butyl (1-cyano-2-phenylethyl)carbamate (99)

Yield 91%. White solid. Mp 156 – 160. ^1H NMR (500 MHz, CDCl_3) δ 7.80 (s, 1H) 7.33 – 7.23 (m, 5H), 4.63 (m, 1H), 3.04 – 3.09 (m, 2H), 1.35 (s, 9H). ESI-MS (+) Calc. for $[\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_2]$ 246.14, found: 245.30 $[\text{M}+\text{H}]^+$. HPLC (protocol A, 30:70 ACN: water): t_R (min) = 55.80. Purity > 99.9 %.

Synthesis of compounds **100** – **119** was performed by coupling the corresponding trifluoroethylamine carboxylic acid (**91** – **98**) with 1-aminocyclopropanecarbonitrile or with (S)-2-amino-3-phenylpropanenitrile (general procedure for amide synthesis, method A). (S)-2-amino-3-phenylpropanenitrile was prepared by removal of Boc protecting group from compound **99** (general procedure for removal of the Boc protecting group, method A), and it was used directly for the coupling reaction. The purity for all final compounds is over 95%. When necessary, compounds were purified by HPLC equipped with a chiral column.

(S)-N-(1-cyanocyclopropyl)-4-methyl-2-(((R)-2,2,2-trifluoro-1-phenylethyl)amino)pentanamide (100)

Yield 71%. Colorless oil. R_f = 0.5 (ethyl acetate/ *n*-hexane 70:30). $[\alpha]_{\text{D}}^{24}$ = - 108° (MeOH, c = 1.03×10^{-3}). ^1H NMR (400 MHz, CDCl_3) δ 7.66 (s, 1H), 7.45 – 7.39 (m, 3H), 7.33-7.31 (m, 2H), 3.80 (q, J = 7.37 Hz, 1H), 3.07 (dd, J = 9.77, 3.64 Hz, 1H), 1.99 (s, 1H), 1.64 – 1.42 (m, 5H), 1.41 – 1.35 (m, 1H), 1.24 – 1.19 (m, 2H), 0.85 (d, J = 6.28 Hz, 3H), 0.57 (d, J = 7.17 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.8, 133.3, 129.6,

129.1, 128.0, 124.9 (q, $J = 230$ Hz), 119.8, 63.8 (q, $J = 30$ Hz) 59.0, 42.6, 24.6, 23.2, 23.1, 20.9, 20.1, 16.9, 16.4. FT-IR (KBr, cm^{-1}) 3319, 2959, 2253, 1686, 1525, 1331, 1263, 1173, 1107, 887, 704. HRMS (+) Calc. for $[\text{C}_{18}\text{H}_{23}\text{F}_3\text{N}_3\text{O}]^+$ 354.17877, found: 354.17926 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 20.85. After HPLC purification *d.e.* = 96.0%.

(S)-*N*-(1-cyanocyclopropyl)-4-methyl-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)pentanamide (**101**)

Yield 71%. White solid. $R_f = 0.5$ (ethyl acetate/ *n*-hexane 70:30). Mp. 144 – 146 °C. $[\alpha]_D^{24} = +8^\circ$ (MeOH, $c = 1.02 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.42 – 7.37 (m, 3H), 7.34 – 7.32 (m, 2H), 7.15 (s, 1H), 4.03 (q, $J = 7.37$ Hz, 1H), 3.35 (dd, $J = 8.95, 4.75$ Hz, 1H), 2.12 (s, 1H), 1.76 (ddq, $J = 13.00, 8.74, 6.53$ Hz, 1H), 1.60 (ddd, $J = 13.70, 8.81, 4.78$ Hz, 1H), 1.49 – 1.36 (m, 3H), 1.00 – 0.96 (m, 1H), 0.95 (d, $J = 2.50$ Hz, 3H), 0.94 (d, $J = 2.56$ Hz, 3H), 0.83 – 0.77 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.8, 134.1, 129.5, 129.0, 125.3 (q, $J = 220$ Hz), 119.6, 64.2, 63.8 (q, $J = 28.7$ Hz), 42.6, 24.8, 23.1, 21.7, 19.9, 16.8, 16.2. FT-IR (KBr, cm^{-1}) 3319, 2959, 2253, 1686, 1525, 1331, 1263, 1173, 1107, 887, 704. HRMS (+) Calc. for $[\text{C}_{18}\text{H}_{23}\text{F}_3\text{N}_3\text{O}]^+$ 354.17877, found: 354.17926 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 12.91. After HPLC purification *d.e.* > 99.9%

(S)-2-(((*R*)-1-([1,1'-biphenyl]-4-yl)-2,2,2-trifluoroethyl)amino)-*N*-(1-cyano-cyclopropyl)-4-methylpentanamide (**102**)

Yield 78%. White solid. $R_f = 0.8$ (ethyl acetate/ *n*-hexane 70:30). Mp. 149 – 151 °C. $[\alpha]_D^{24} = -126^\circ$ (MeOH, $c = 1.21 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.63 – 7.66 (m, 3H), 7.59 – 7.61 (m, 2H), 7.45 – 7.49 (m, 2H), 7.37 – 7.42 (m, 3H), 3.88 (m, 1H), 3.12 (dd, $J = 9.6, 2.6$ Hz, 1H), 1.50 – 1.64 (m, 4H), 1.40 – 1.44 (m, 1H), 1.21 – 1.32 (m, 3H), 0.88 (d, $J = 5.9$ Hz, 3H), 0.61 (d, $J = 6.0$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.9, 142.6, 140.0, 132.1, 128.9, 128.5, 127.8, 127.7, 127.1, 125.0 (q, $J = 220$ Hz), 119.8, 63.5 (q, $J = 29.3$ Hz), 59.0, 42.7, 24.7, 23.2, 21.0, 20.1, 16.9, 16.4. HRMS (+) Calc. for $[\text{C}_{24}\text{H}_{27}\text{F}_3\text{N}_3\text{O}]^+$ 430.21007, found: 430.21014 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 55:45): t_R (min) = 17.63. After HPLC purification *d.e.* > 99.9%.

(S)-2-(((*S*)-1-([1,1'-biphenyl]-4-yl)-2,2,2-trifluoroethyl)amino)-*N*-(1-cyano-cyclopropyl)-4-methylpentanamide (**103**)

Yield 78%. White solid. $R_f = 0.8$ (ethyl acetate/ *n*-hexane 70:30). Mp. 200 – 204 °C. $[\alpha]_D^{24} = +38^\circ$ (MeOH, $c = 1.21 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.64 – 7.58 (m, 4H), 7.48 – 7.37 (m, 5H), 7.20 (s, 1H), 4.11 (q, $J = 7.3$ Hz, 1H), 3.40 (dd, $J = 8.8, 4.8$ Hz, 1H), 1.75 – 1.85 (m, 1H), 1.60 – 1.66 (m, 1H), 1.39 – 1.53 (m, 3H), 0.99 – 1.04 (m, 1H), 0.98 (d, $J = 3.2$ Hz, 3H), 0.97 (d, $J = 3.3$ Hz, 3H), 0.84 – 0.92 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.8, 142.5, 140.0, 132.9, 128.9, 128.7, 127.8, 127.7, 127.1, 123.9, 119.6, 63.5 (q, $J = 28.9$ Hz), 60.0, 42.6, 24.8, 23.0, 21.8, 19.9, 16.8, 16.3. ^{19}F NMR (376 MHz, CDCl_3) δ -73.65 (d, $J = 7.1$ Hz). HRMS (+) Calc. for $[\text{C}_{24}\text{H}_{27}\text{F}_3\text{N}_3\text{O}]^+$

430.21007, found: 430.21014 $[M+H]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 20.85. After HPLC purification *d.e.* > 99.9%.

(S)-*N*-(1-cyanocyclopropyl)-3-phenyl-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**104**)

Yield 92%. Colorless oil. R_f = 0.5 (ethyl acetate/ *n*-hexane 30:70). $[\alpha]_D^{24}$ = -60° (MeOH, $c = 1.07 \times 10^{-3}$). HRMS (+) Calc. for $[C_{21}H_{21}F_3N_3O]^+$ 388.16312, found: 388.16379 $[M+H]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 21.11. (BURTOLOSO et al., 2017)

(S)-*N*-(1-cyanocyclopropyl)-3-phenyl-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**105**)

Yield 92%. White solid. R_f = 0.5 (ethyl acetate/ *n*-hexane 30:70). Mp. 141 – 143 $^\circ\text{C}$. $[\alpha]_D^{24}$ = $+45^\circ$ (MeOH, $c = 1.07 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.19 – 7.43 (m, 10H), 3.99 (q, $J = 7.19\text{ Hz}$, 1H), 3.43 (m, 1H), 3.11 (dd, $J = 13.89, 5.43\text{ Hz}$, 1H), 3.06 (dd, $J = 13.89, 6.83\text{ Hz}$, 1H), 2.03 (s, 1H), 1.42 – 1.51 (m, 2H), 1.00 – 1.04 (m, 1H), 0.86 – 0.92 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.4135.8, 133.1, 129.6, 129.3, 129.1, 129.0, 128.6, 127.5, 124.9 (q, $J = 220\text{ Hz}$), 119.5, 63.5 (q, $J = 30\text{ Hz}$), 60.8, 37.9, 19.9, 16.7, 16.4. FT-IR (KBr, cm^{-1}): 3329, 3246, 3032, 2237, 1649, 1539, 1499, 1456, 1263, 1171, 1126, 850, 742, 567 cm^{-1} . HRMS (+) Calc. for $[C_{21}H_{21}F_3N_3O]^+$ 388.16312, found: 388.16379 $[M+H]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 17.57. After HPLC purification *d.e.* > 99.9%.

(S)-2-(((R)-1-(4-bromophenyl)-2,2,2-trifluoroethyl)amino)-N-(1-cyanocyclopropyl)-3-phenylpropanamide (**106**)

Yield 78%. White solid. $R_f = 0.3$ (ethyl acetate/ *n*-hexane 30:70). Mp 130 – 133 °C. . $[\alpha]_D^{24} = -51^\circ$ (MeOH, $c = 1.21 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.50 – 7.54 (m, 2H), 7.29 – 7.38 (m, 3H), 7.12 – 7.19 (m, 5H), 3.97 (m, 1H), 3.39 (q, $J = 6.24\text{ Hz}$, 1H), 3.08 (dd, $J = 3.6, 1.9\text{ Hz}$, 1H), 3.05 (dd, $J = 11.8, 4.6\text{ Hz}$, 1H), 2.19 (s, 1H), 1.45 – 1.52 (m, 2H), 0.92 – 1.06 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.2, 135.7, 132.2, 132.0, 130.2, 129.2, 129.1, 127.5, 124.6 (q, $J = 230\text{ Hz}$), 123.5, 119.4, 62.82 (q, $J = 29.0\text{ Hz}$), 60.7, 38.0, 20.0, 16.7, 16.5. FT-IR (KBr, cm^{-1}): 3339, 3028, 2243, 1686, 1491, 1263, 1170, 1124, 1010, 814, 727, 702 HRMS (+) Calc. for $[\text{C}_{21}\text{H}_{19}\text{BrF}_3\text{N}_3\text{O}]^+$ 466.07364, found: 466.07471 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 12.55. After HPLC purification *d.e.* > 99%.

(S)-2-(((R)-1-(3-chlorophenyl)-2,2,2-trifluoroethyl)amino)-N-(1-cyanocyclopropyl)-3-phenylpropanamide (**107**)

Yield 73%. Colorless oil. $R_f = 0.5$ (ethyl acetate/ *n*-hexane 30:70). $[\alpha]_D^{24} = +77^\circ$ (MeOH, $c = 1.21 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.66 (s, 1H), 7.25 – 7.31 (m, 4H), 7.14 (t, $J = 7.9\text{ Hz}$, 1H), 7.00 – 6.97 (m, 2H), 6.78 (s, 1H), 6.70 (d, $J = 7.7\text{ Hz}$, 1H), 3.72 (s, 1H), 3.20 – 3.17 (m, 2H), 2.63 (dd, $J = 13.5, 9.9\text{ Hz}$, 1H), 2.08 (s, 1H), 1.55 – 1.65 (m, 2H), 1.17 – 1.32 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.4, 135.5, 134.8, 134.2, 130.0, 129.6, 129.1, 128.8, 127.8, 127.7, 126.2, 125.9, 123.1, 119.6, 63.6, 63.3, 63.0, 62.7, 61.7, 39.2, 20.1, 16.9, 16.4. FT-IR (KBr, cm^{-1}): 3336, 3057, 3030, 2250, 1683, 1531, 1475, 1354, 1138, 1101, 897, 790, 761, 700, 514. HRMS (+) Calc. for

$[\text{C}_{21}\text{H}_{20}\text{F}_3\text{N}_3\text{ClO}]^+$ 422.12415, found: 422.12399 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 25.31. After HPLC purification *d.e.* > 99%.

(S)-N-(1-cyanocyclopropyl)-3-phenyl-2-(((S)-2,2,2-trifluoro-1-(4-methoxyphenyl)ethyl)amino)propanamide (**108**)

Yield 90%. Colorless oil. R_f = 0.5 (ethyl acetate/ *n*-hexane 30:70). $[\alpha]_{\text{D}}^{24} = +59.35^\circ$ (MeOH, $c = 1.23 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.42 – 7.36 (m, 3H), 7.28 – 7.27 (m, 3H), 7.13 – 7.10 (m, 2H), 6.91 – 6.88 (m, 2H), 4.02 (q, $J = 7.2$ Hz, 1H), 3.04 (dd, $J = 12.4, 4.6$ Hz, 1H), 3.00 (dd, $J = 12.4, 4.1$ Hz, 1H), 2.19 (s, 1H), 1.40 – 1.50 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.6, 158.9, 133.1, 130.3, 129.6, 129.0, 128.6, 127.5, 126.4, 123.6, 119.5, 114.4, 63.31 (q, $J = 28.8$ Hz), 55.3, 36.9, 19.9, 16.7, 16.4. FT-IR (KBr, cm^{-1}): 3273, 3038, 2237, 1672, 1610, 1533, 1512, 1265, 1145, 1039, 873, 794, 561. HRMS (+) Calc. for $[\text{C}_{22}\text{H}_{23}\text{F}_3\text{N}_3\text{O}_2]^+$ 418.17369, found: 418.17435 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 18.9. After HPLC purification *d.e.* > 99%.

(S)-N-(1-cyanocyclopropyl)-3-(1H-indol-3-yl)-2-(((R)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**109**)

Yield 82%. Colorless oil. R_f = 0.5 (ethyl acetate/ *n*-hexane 50:50). ^1H NMR (400 MHz, CDCl_3) δ 8.20 (s, 1H), 7.63 (s, 1H), 7.30 – 7.25 (m, 2H), 7.11 (m, 2H), 6.99 – 6.90 (m, 3H), 6.85 (d, $J = 2.0$ Hz, 1H), 6.68 (d, $J = 7.2$ Hz, 1H), 3.69 (q, $J = 7.6$ Hz, 1H), 3.30

(dd, $J = 9.2, 4.4$ Hz, 1H), 3.20 (dd, $J = 14.4, 4.4$ Hz, 1H), 2.87 (dd, $J = 14.4, 9.2$ Hz, 1H), 1.78 (br s, 1H), 1.50 – 1.49 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.7, 136.5, 132.5, 129.2, 128.6, 127.9, 127.1, 127.1, 125.0 (q, $J = 279.1$ Hz), 123.5, 122.5, 119.9, 119.9, 118.4, 111.5, 109.7, 63.9 ($J = 28.9$ Hz), 61.0, 29.3, 17.0, 16.5. HRMS (+) Calc. for $[\text{C}_{23}\text{H}_{21}\text{F}_3\text{N}_4\text{O}]^+$ 427.1746, found: 427.1741 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 18.6. After HPLC purification *d.e.* > 99%.

(R)-*N*-(1-cyanocyclopropyl)-3-(1*H*-indol-3-yl)-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**110**)

Yield 74%. Colorless oil. $R_f = 0.5$ (ethyl acetate/ *n*-hexane 50:50). ^1H NMR (400 MHz, CDCl_3) δ 8.20 (s, 1H), 7.61 (s, 1H), 7.31 – 7.26 (m, 2H), 7.12 (m, 2H), 6.95 – 6.89 (m, 3H), 6.86 (d, $J = 2.0$ Hz, 1H), 6.68 (d, $J = 7.2$ Hz, 1H), 3.69 (q, $J = 7.6$ Hz, 1H), 3.32 (dd, $J = 9.1, 4.4$ Hz, 1H), 3.24 (dd, $J = 14.3, 4.4$ Hz, 1H), 2.88 (dd, $J = 14.4, 9.2$ Hz, 1H), 1.80 (br s, 1H), 1.54 – 1.51 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.7, 136.4, 132.5, 129.2, 128.9, 127.2, 127.3, 127.2, 125.0 (q, $J = 279.1$ Hz), 123.5, 122.3, 119.9, 119.9, 118.4, 111.2, 109.7, 63.9 ($J = 28.9$ Hz), 61.1, 29.2, 17.0, 16.7. HRMS (+) Calc. for $[\text{C}_{23}\text{H}_{21}\text{F}_3\text{N}_4\text{O}]^+$ 427.1746, found: 427.1741 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 16.73. After HPLC purification *d.e.* > 99%.

(S)-*N*-(((*S*)-1-cyano-2-phenylethyl)-4-methyl-2-(((*R*)-2,2,2-trifluoro-1-phenylethyl)amino)pentanamide (**111**)

Yield 76%. Colorless oil. $R_f = 0.7$ (ethyl acetate/ *n*-hexane 30:70). Mp. 152 – 154 °C. $[\alpha]_D^{24} = -115^\circ$ (MeOH, $c = 1.21 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.52 (d, $J = 8.8$

Hz, 1H), 7.26 – 7.41 (m, 10H), 5.12 (dt, $J = 8.8, 7.0$ Hz, 1H), 3.74 – 3.77 (m, 1H), 3.15 (d, $J = 7.0$ Hz, 2H), 3.02 (dd, $J = 9.8, 3.9$ Hz, 1H), 1.93 (d, $J = 9.1$ Hz, 1H), 1.32 – 1.44 (m, 2H), 1.19 – 1.25 (m, 1H), 0.80 (d, $J = 6.4$ Hz, 3H), 0.49 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.4, 133.8, 133.3, 129.5, 129.3, 129.0, 129.0, 128.0, 127.9, 123.4, 117.9, 63.5 (q, $J = 29.2$ Hz), 58.4, 42.7, 41.1, 38.9, 24.6, 23.1, 20.8. FT-IR (KBr, cm^{-1}): 3331, 3360, 2966, 2934, 2245, 1672, 1516, 1497, 1267, 1107, 887, 698. HRMS (+) Calc. for $[\text{C}_{23}\text{H}_{27}\text{F}_3\text{N}_3\text{O}]^+$ 418.17369, found: 418.17877 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 14.02. After HPLC purification *d.e.* > 99%.

(S)-*N*-(((*S*)-1-cyano-2-phenylethyl)-4-methyl-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)pentanamide (**112**)

Yield 76%. White solid. $R_f = 0.7$ (ethyl acetate/ *n*-hexane 30:70). Mp. 125 – 129 °C. $[\alpha]_D^{24} = -26^\circ$ (MeOH, $c = 1.21 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.39 – 7.39 (m, 6H), 7.22 – 7.19 (m, 4H), 7.11 (d, $J = 8.77$ Hz, 1H), 4.90 – 5.00 (m, 1H), 4.00 (q, $J = 7.20$ Hz, 1H), 3.32 (dd, $J = 8.87, 4.73$ Hz, 1H), 3.00 (dd, $J = 11.50, 4.37$ Hz, 1H), 2.97 (dd, $J = 11.50, 4.28$ Hz, 1H), 1.99 (s, 1H), 1.70 (ddq, $J = 13.04, 8.79, 6.54$ Hz, 1H), 1.51 (ddd, $J = 13.75, 8.85, 4.76$ Hz, 1H), 1.34 (ddd, $J = 14.21, 8.92, 5.58$ Hz, 1H), 0.91 (dd, $J = 9.59, 6.57$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.4, 133.7, 133.5, 129.5, 129.3, 129.1, 129.0, 128.3, 128.0, 124.2, 117.7, 63.6 (d, $J = 28.6$ Hz), 59.2, 42.6, 40.8, 38.8, 24.8, 23.0, 21.8. FT-IR (KBr, cm^{-1}) 3331, 3360, 2966, 2934, 2245, 1672, 1516, 1497, 1267, 1107, 887, 698. HRMS (+) Calc. for $[\text{C}_{23}\text{H}_{27}\text{F}_3\text{N}_3\text{O}]^+$ 418.17369, found: 418.17877 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 13.67. After HPLC purification *d.e.* > 99%.

(*S*)-2-(((*R*)-1-([1,1'-biphenyl]-4-yl)-2,2,2-trifluoroethyl)amino)-*N*-((*S*)-1-cyano-2-phenylethyl)-4-methylpentanamide (**113**)

Yield 81%. White solid. $R_f = 0.8$ (ethyl acetate/ *n*-hexane 10:90). Mp. 173 – 176 °C. $[\alpha]_D^{24} = -113^\circ$ (MeOH, $c = 1.1 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.57 – 7.64 (m, 4H), 7.43 – 7.48 (m, 3H), 7.27 – 7.41 (m, 8H), 5.13 (dt, $J = 8.8, 7.0$ Hz, 1H), 3.82 (q, $J = 7.6$ Hz, 1H), 3.16 (d, $J = 7.0$ Hz, 2H), 3.07 (dd, $J = 9.7, 4.0$ Hz, 1H), 1.34 – 1.50 (m, 2H), 1.20 – 1.28 (m, 1H), 0.82 (d, $J = 6.3$ Hz, 3H), 0.53 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.1, 142.5, 140.0, 133.8, 132.1, 129.3, 129.0, 128.9, 127.9, 127.8, 127.7, 127.1, 123.4, 117.9, 63.2 (q, $J = 63.19$ Hz), 58.5, 42.7, 41.0, 38.9, 24.6, 23.1, 20.9. HRMS (+) Calc. for $[\text{C}_{29}\text{H}_{31}\text{F}_3\text{N}_3\text{O}]^+$ 494.23895, found: 494.24137 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 24.2. After HPLC purification *d.e.* > 99%.

(*S*)-2-(((*S*)-1-([1,1'-biphenyl]-4-yl)-2,2,2-trifluoroethyl)amino)-*N*-((*S*)-1-cyano-2-phenylethyl)-4-methylpentanamide (**114**)

Yield 85%. White solid. $R_f = 0.8$ (ethyl acetate/ *n*-hexane 10:90). Mp. 164 – 166 °C. $[\alpha]_D^{24} = -21^\circ$ (MeOH, $c = 1.1 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.60 – 7.57 (m, 4H), 7.47 – 7.44 (m, 2H), 7.42 – 7.29 (m, 6H), 7.23 – 7.21 (m, 2H), 7.12 (d, $J = 8.8$ Hz, 1H), 5.04 (dt, $J = 8.9, 6.6$ Hz, 1H), 4.07 (q, $J = 7.2$ Hz, 1H), 3.37 (dd, $J = 8.9, 4.7$ Hz, 1H), 3.02 (d, $J = 6.64$ Hz, 2H), 1.68 – 1.77 (m, 2H), 1.52 – 1.57 (m, 1H), 1.35 – 1.40 (m, 1H), 0.96 (d, $J = 6.5$ Hz, 3H), 0.94 (d, $J = 6.6$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.3, 142.4, 140.2, 133.7, 132.3, 129.3, 129.0, 128.9, 128.8, 128.7, 128.0, 127.8, 127.7, 127.2, 124.2, 117.7, 63.4 (q, $J = 25$ Hz), 59.2, 42.6, 40.8, 38.8, 24.9, 23.1, 21.8. FT-IR (KBr, cm^{-1}) 3313, 2954, 1672, 1531, 1494, 1332, 1265, 1174, 736, 700. HRMS

(+) Calc. for $[C_{29}H_{31}F_3N_3O]^+$ 494.23895, found: 494.24137 $[M+H]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 15.83. After HPLC purification *d.e.* = 95%.

(2*S*)-2-[[*(1R)*-1-[[*1,1'*-biphenyl]-4-yl]-2,2,2-trifluoroethyl]amino]-*N*-[(*1S*)-1-cyano-2-phenylethyl]-3-phenylpropanamide (**115**)

Yield 64%. Colorless oil. R_f = 0.6 (ethyl acetate/ *n*-hexane 10:90). 1H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, J = 8.5 Hz, 1H), 7.55 – 7.53 (m, 2H), 7.47 – 7.44 (m, 2H), 7.41 – 7.35 (m, 6H), 7.29 – 7.23 (m, 5H), 6.98 (d, J = 7.0 Hz, 2H), 6.76 (d, J = 8.0 Hz, 2H), 5.14 (q, J = 7.5 Hz, 1H), 3.73 (d, J = 8.0 Hz, 1H), 3.24 – 3.06 (m, 4H), 2.40 (dd, J = 14.0, 10.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1, 142.1, 140.1, 135.8, 133.8, 129.4, 129.1, 129.0, 129.0, 128.9, 128.1, 128.0, 127.7, 127.4, 127.4, 127.1, 117.8, 71.6, 64.3, 63.0, 61.1, 41.2, 39.2, 38.9. HRMS (+) Calc. for $[C_{29}H_{31}F_3N_3O]^+$ 494.23895, found: 494.24137 $[M+H]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 22.94. After HPLC purification *d.e.* = 95%.

(2*S*)-*N*-[(*1S*)-1-cyano-2-phenylethyl]-3-(4-methoxyphenyl)-2-[[*(1R)*-2,2,2-trifluoro-1-phenylethyl]amino]propanamide (**116**)

Yield 75%. Colorless oil. R_f = 0.6 (ethyl acetate/ *n*-hexane 30:70). $[\alpha]_D^{24}$ = -51° (MeOH, c = 1.1×10^{-3}). 1H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, J = 9.2 Hz, 1H), 7.42 – 7.27 (m,

6H), 7.19 – 7.14 (m, 2H), 6.89 – 6.86 (m, 2H), 6.80 – 6.77 (m, 2H), 6.64 (d, $J = 7.5$ Hz, 2H), 5.30 (ddd, $J = 9.1, 8.3, 6.8$ Hz, 1H), 3.81 (s, 3H), 3.41 – 3.36 (m, 1H), 3.28 (dd, $J = 14.2, 6.8$ Hz, 1H), 3.14 (dd, $J = 14.2, 8.2$ Hz, 1H), 3.06 – 3.10 (m, 2H), 2.58 (dd, $J = 14.7, 10.9$ Hz, 1H), 2.07 (d, $J = 10.4$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.1, 159.0, 133.9, 132.2, 130.0, 129.2, 129.1, 129.0, 128.7, 127.9, 127.8, 127.6, 126.1, 123.3, 118.1, 114.4, 63.5, 63.2, 62.9, 62.6, 61.1, 55.3, 40.6, 39.0, 38.3. FT-IR (KBr, cm^{-1}) 3352, 3311, 3030, 2839, 2937, 2360, 2245, 1682, 1612, 1514, 1456, 1252, 1126, 885, 750, 696, 548. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{27}\text{F}_3\text{N}_3\text{O}_2]^+$ 482.20499, found: 482.20566 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 28.16. After HPLC purification *d.e.* > 99%.

(S)-*N*-((*R*)-1-cyano-2-phenylethyl)-3-phenyl-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**117**)

Yield 78%. White solid. $R_f = 0.8$ (ethyl acetate/ *n*-hexane 30:70). Mp. 92 – 96 °C. $[\alpha]_D^{24} = +75^\circ$ (MeOH, $c = 1.4 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.40 – 7.29 (m, 10H), 7.20 – 7.12 (m, 6H), 4.98 (dt, $J = 8.1, 7.0$ Hz, 1H), 3.95 (m, 1H), 3.38 (dd, $J = 12.6, 6.8$ Hz, 1H), 3.11 (dd, $J = 13.9, 5.3$ Hz, 1H), 3.06 (dd, $J = 13.9, 6.7$ Hz, 1H), 2.99 (dd, $J = 12.9, 6.0$ Hz, 1H), 2.95 (dd, $J = 12.9, 6.0$ Hz, 1H), 2.11 (dd, $J = 7.2, 4.7$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.2, 135.8, 133.7, 132.7, 129.6, 129.3, 129.2, 129.1, 129.1, 128.9, 128.7, 128.0, 127.5, 126.0, 123.8, 117.8, 63.4, 63.1, 62.9, 62.7, 59.8, 41.3, 38.4, 37.6. FT-IR (KBr, cm^{-1}) 3360, 3302, 2966, 2935, 2245, 1672, 1514, 1497, 1373, 1267, 1167, 1124, 1109, 887, 756, 696. HRMS (+) Calc. for $[\text{C}_{26}\text{H}_{25}\text{F}_3\text{N}_3\text{O}]^+$ 452.19442, found: 452.19458 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 18.10. After HPLC purification *d.e.* > 99%.

(*S*)-*N*-((*R*)-1-cyano-2-phenylethyl)-3-(4-methoxyphenyl)-2-(((*R*)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**118**)

Yield 75%. Colorless oil. $R_f = 0.5$ (ethyl acetate/ *n*-hexane 30:70). $[\alpha]_D^{24} = -51^\circ$ (MeOH, $c = 1.4 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.63 (d, $J = 9.2$ Hz, 1H), 7.42 – 7.27 (m, 6H), 7.19 – 7.14 (m, 2H), 6.89 – 6.86 (m, 2H), 6.80 – 6.77 (m, 2H), 6.64 (d, $J = 7.5$ Hz, 2H), 5.30 (ddd, $J = 9.1, 8.3, 6.8$ Hz, 1H), 3.81 (s, 3H), 3.41 – 3.36 (m, 1H), 3.28 (dd, $J = 14.2, 6.8$ Hz, 1H), 3.14 (dd, $J = 14.2, 8.2$ Hz, 1H), 3.10 – 3.06 (m, 2H), 2.58 (dd, $J = 14.7, 10.9$ Hz, 1H), 2.07 (d, $J = 10.4$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.1, 159.0, 133.9, 132.2, 130.0, 129.2, 129.1, 129.0, 128.7, 127.9, 127.8, 127.6, 126.1, 123.3, 118.1, 114.4, 63.5, 63.2, 62.9, 62.6, 61.1, 55.3, 40.6, 39.0, 38.3. FT-IR (KBr, cm^{-1}) 3352, 3311, 3030, 2839, 2937, 2360, 2245, 1682, 1612, 1514, 1456, 1252, 1126, 885, 750, 696, 548. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{27}\text{F}_3\text{N}_3\text{O}_2]^+$ 482.20499, found: 482.20566 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 28.17. After HPLC purification *d.e.* > 99%.

(*S*)-*N*-((*R*)-1-cyano-2-phenylethyl)-3-(4-methoxyphenyl)-2-(((*S*)-2,2,2-trifluoro-1-phenylethyl)amino)propanamide (**119**)

Yield 75%. Colorless oil. $R_f = 0.5$ (ethyl acetate/ *n*-hexane 30:70). $[\alpha]_D^{24} = +48^\circ$ (MeOH, $c = 1.4 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.41 – 7.31 (m, 7H), 7.21 – 7.09 (m, 6H), 6.92 – 6.89 (m, 2H), 4.98 (dd, $J = 15.1, 7.0$ Hz, 1H), 4.01 (q, $J = 7.01$ Hz, 1H), 3.81 (s, 3H), 3.35 (t, $J = 5.8$ Hz, 1H), 3.07 – 2.94 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.4, 159.0, 133.8, 132.8, 130.4, 129.6, 129.2, 129.1, 128.9, 128.7, 128.0, 127.5, 126.1,

123.8, 117.8, 114.5, 63.2 (q, $J = 28.7$ Hz), 59.8, 55.3, 41.3, 38.4, 36.5. FT-IR (KBr, cm^{-1}) 3352, 3311, 3030, 2839, 2937, 2360, 2245, 1682, 1612, 1514, 1456, 1252, 1126, 885, 750, 696, 548. HRMS (+) Calc. for $[\text{C}_{27}\text{H}_{27}\text{F}_3\text{N}_3\text{O}_2]^+$ 482.20499, found: 482.20566 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 19.87. After HPLC purification *d.e.* > 99%.

Compounds **120** and **121** were synthesized by a Suzuki cross-coupling reaction of the aryl bromide intermediates (general procedure for Suzuki cross-coupling reaction)

(*S*)-*N*-(1-cyanocyclopropyl)-4-methyl-2-(((*R*)-2,2,2-trifluoro-1-(4'-fluoro-[1,1'-biphenyl]-4-yl)ethyl)amino)pentanamide (**120**)

Yield 81%. White solid. $R_f = 0.7$ (ethyl acetate/ *n*-hexane 30:70). Mp 145 – 147 °C. $[\alpha]_D^{24} = -119^\circ$ (MeOH, $c = 1.4 \times 10^{-3}$). ^1H NMR (400 MHz, CDCl_3) δ 7.64 (s, 1H), 7.60 – 7.53 (m, 4H), 7.41 – 7.38 (m, 2H), 7.17 – 7.13 (m, 2H), 3.88 (q, $J = 7.5$ Hz, 1H), 3.10 (dd, $J = 9.6, 3.5$ Hz, 1H), 2.02 (d, $J = 7.6$ Hz, 1H), 1.64 – 1.48 (m, 4H), 1.47 – 1.41 (m, 1H), 1.32 – 1.20 (m, 2H), 0.87 (d, $J = 6.2$ Hz, 3H), 0.61 (d, $J = 6.0$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 174.8, 164.0, 161.5, 141.5, 136.1, 136.1, 132.2, 128.7, 128.6, 128.6, 127.6, 126.3, 123.5, 119.8, 115.9, 115.7, 63.5 (q, $J = 29.3$ Hz), 59.0, 42.7, 24.7, 23.1, 21.0, 20.1, 16.9, 16.4. HRMS (+) Calc. for $[\text{C}_{24}\text{H}_{26}\text{F}_4\text{N}_3\text{O}]^+$ 448.20065, found: 448.20114 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 28.10. After HPLC purification *d.e.* > 99%.

(*S*)-*N*-((*S*)-1-cyano-2-phenylethyl)-4-methyl-2-(((*S*)-2,2,2-trifluoro-1-(4-(pyrimidin-5-yl)phenyl)ethyl)amino)pentanamide (**121**)

Yield 60%. White solid. R_f = 0.7 (ethyl acetate/ *n*-hexane 50:50). Mp 59 – 61 °C. ^1H NMR (400 MHz, CDCl_3) δ 9.22 (s, 1H), 8.93 (s, 2H), 7.53 (d, J = 8.0 Hz, 2H), 7.37 – 7.37 – 7.31 (m, 5H), 7.23 (d, J = 7.6 Hz, 2H), 7.05 (d, J = 9.2 Hz, 1H), 5.06 – 5.01 (m, 1H), 4.06 (q, 7.2 Hz, 1H), 3.35 – 3.31 (m, 1H), 3.05 – 2.97 (m, 2H), 1.77 – 1.66 (m, 1H), 1.56 – 1.49 (m, 1H), 1.41 – 1.34 (m, 1H), 0.96 – 0.94 (m, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.1, 157.8, 155.0, 135.5, 133.7, 129.5, 129.4, 129.1, 128.1, 127.8, 117.7, 63.66 (d, J = 28.7 Hz), 59.7, 42.9, 40.7, 38.8, 24.9, 23.1, 21.8. ^{19}F NMR (376.28 Hz, CDCl_3): δ -73.49 (d, J = 6.6 Hz). ESI-MS (+) Calc. for $[\text{C}_{27}\text{H}_{29}\text{F}_3\text{N}_5\text{O}]^+$ 496.53, found: 496.21 $[\text{M}+\text{H}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 21.52. After HPLC purification *d.e.* > 99%.

Synthesis of compounds **123** and **124** were performed in two steps starting from Boc-L-leucine. First Boc-L-leucine was coupled with the selected amine (general procedure for amide synthesis, method A) to obtain compounds **21** and **122**. Then after removal of the Boc protecting group (general procedure for removal of the Boc protecting group, method A), the free amines were coupled with benzoic acid (general procedure for amide synthesis, method A).

tert-butyl ((*S*)-1-(((*S*)-1-cyano-2-phenylethyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (**122**)

Yield 92%. White solid. R_f = 0.7 (ethyl acetate/ *n*-hexane 20:80). Mp 137 – 138 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.35 – 7.26 (m, 5H), 7.04 (br s, 1H), 5.08 (m, 1H), 4.90 (d, J

= 6.8 Hz, 1H), 3.06 (m, 2H), 1.65 – 1.60 (m, 2H), 1.45 (s, 9H), 1.28 – 1.24 (m, 2H), 0.93-0.89 (m, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.2, 156.0, 134.0, 129.6, 129.1, 128.1, 118.0, 80.9, 52.9, 41.8, 40.5, 39.1, 28.4, 24.8, 23.0, 22.0. ESI-MS (+) Calc. for $[\text{C}_{20}\text{H}_{29}\text{N}_3\text{O}_3]$ 359.46, found: 382.24 $[\text{M}+\text{Na}]^+$.

(S)-*N*-(1-((1-cyanocyclopropyl)amino)-4-methyl-1-oxopentan-2-yl)benzamide (**123**)

Yield 60%. White solid. R_f = 0.7 (ethyl acetate/ *n*-hexane 70:30). Mp 189 – 191 °C cm^{-1} . ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.95 (s, 1H), 8.59 (d, J = 10.5 Hz, 1H), 7.91 – 7.88 (m, 2H), 7.56 – 7.51 (m, 1H), 7.60 (m, 1H), 7.48 – 7.44 (m, 2H), 4.45 – 4.50 (m, 1H), 1.74 – 1.60 (m, 2H), 1.52 – 1.49 (m, 1H), 1.48 – 1.45 (m, 1H), 1.12 – 1.09 (m, 2H), 0.97 (d, J = 8.2 Hz, 3H), 0.86 (d, J = 8.1 Hz, 3H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 174.17, 166.81, 134.35, 131.77, 128.59, 128.03, 121.27, 78.36, 51.87, 27.19, 26.93, 24.86, 23.44, 16.19. FT-IR (KBr, cm^{-1}): 3265, 3045, 2959, 2931, 2872, 2243, 1683, 1540, 1541, 1490, 1301, 1327. ESI-MS (+) Calc. for $[\text{C}_{17}\text{H}_{21}\text{N}_3\text{O}_2]$ 299.36, found: 300.67 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 45:55): t_R (min) = 9.89

N-(((*S*)-1-(((*S*)-1-cyano-2-phenylethyl)amino)-4-methyl-1-oxopentan-2-yl)benzamide (**124**)

Yield 79%. White solid. R_f = 0.5 (ethyl acetate/ *n*-hexane 50:50). Mp 203 – 205 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.84 (d, J = 9.5 Hz 1H), 8.48 (d, J = 10 Hz, 1H), 7.92 – 7.90 (m, 2H), 7.57 – 7.53 (m, 1H), 7.50 – 7.46 (m, 2H), 7.33 – 7.22 (m, 5H), 4.54 – 4.48 (m, 1H), 3.10 (d, J = 10 Hz, 2H), 1.72 – 1.59 (m, 2H), 1.48 – 1.41 (m, 1H), 0.9 (m,

2H), 0.90 (d, $J = 8.1$ Hz, 3H), 0.86 (d, $J = 8.3$ Hz, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.76, 166.75, 135.92, 134.33, 131.76, 129.82, 128.77, 128.56, 128.01, 127.50, 119.41, 51.95, 42.14, 37.57, 24.79, 23.38, 21.74. FT-IR (KBr, cm^{-1}): 3286, 3059, 2958, 2931, 2872, 2850, 2245, 1667, 1652, 1529, 1490, 1327, 1246, 1089. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_2]$ 363.45.36, found: 386.18 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 45:55): t_R (min) = 17.82.

8.1.15. Synthesis and characterization of compounds presented in chapter V

Scheme 16- General synthetic scheme for compounds present in chapter VI.

Reagents and conditions. a) trifluoroacetic anhydride, CH₂Cl₂; b) i) formic acid, rt, 20 h, (ii) corresponding (L)-amino acid, HATU, DIPEA, DMF, rt, 22 h; b) formic acid, rt, 18 h; c) (i) formic acid, rt, 20 h, (ii) corresponding sulfonyl chloride, DIPEA, CH₂Cl₂, 0 °C to rt, 20 h. Source: Own author.

Table 11- List of Nequimed codes of compounds described in chapter V.

Number	Nequimed Code
125	Neq0781
126	Neq0782
127	Neq0831
128	Neq0832
129	Neq0827

Number	Nequimed Code
130	Neq0780
131	Neq0830
132	Neq0848
133	Neq0838
134	Neq0829
135	Neq0936
136	Neq0779
137	Neq0934
138	Neq0935
139	Neq0933

Synthesis and characterization of compounds **122** and **21** have been already described above.

(S)-*N*-((*S*)-1-cyano-2-phenylethyl)-4-methyl-2-(phenylsulfonamido)pentanamide (**125**)

Yield 77%. White solid. R_f = 0.6 (ethyl acetate/ *n*-hexane 70:30). Mp. 130 – 131 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 8.87 (d, J = 9.5 Hz, 1H), 8.08 (d, J = 10.0 Hz, 1H), 7.78 – 7.76 (m, 2H), 7.65 – 7.56 (m, 2H), 7.38 – 7.26 (m, 5H), 4.71 – 4.68 (m, 1H), 3.70 – 3.67 (m, 1H), 3.00 – 2.87 (m, 2H), 1.45 – 1.39 (m, 1H), 1.22 – 1.15 (m, 2H), 0.78 (d, J = 8.5 Hz, 3H), 0.63 (d, J = 8.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.58, 141.28, 135.73, 132.78, 129.79, 129.36, 128.88, 127.63, 126.97, 119.05, 54.83, 42.00, 41.73, 37.52, 24.14, 23.06, 21.64. ESI-MS (+) Calc. for $[\text{C}_{21}\text{H}_{25}\text{N}_3\text{O}_3\text{S}]$ 399.5, found: 422.2 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 22.34. Purity: 99%.

(S)-2-(3-chlorophenylsulfonamido)-*N*-((*S*)-1-cyano-2-phenylethyl)-4-methylpentanamide (**126**)

Yield 65%. White solid. R_f = 0.4 (ethyl acetate/ *n*-hexane 70:30). Mp. 139 – 141 °C. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 8.95 (d, J = 7.5 Hz, 1H), 8.33 (d, J = 8.5 Hz, 1H), 7.78 (t, J = 2.0 Hz, 1H), 7.72 – 7.62 (m, 1H), 7.58 (t, J = 8.0, 1H), 7.36 – 7.23 (m, 5H), 4.69 – 4.65 (m, 1H), 3.74 – 3.70 (m, 1H), 3.00 – 2.87 (m, 2H), 1.50 – 1.45 (m, 1H), 1.34 – 1.18 (m, 2H), 0.79 (d, J = 8.5 Hz, 3H), 0.68 (d, J = 8.5 Hz, 3H). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) δ 171.35, 143.25, 135.63, 133.97, 132.76, 131.52, 129.77, 128.71, 127.69, 126.72, 125.61, 118.92, 54.77, 42.09, 41.73, 37.53, 24.20, 23.10, 21.60. ESI-MS (+) Calc. for $[\text{C}_{21}\text{H}_{24}\text{ClN}_3\text{O}_3\text{S}]$ 433.95, found: 456.1 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 20.45. Purity: 95.5%.

(S)-2-([1,1'-biphenyl]-3-yl)sulfonamido)-*N*-([*(S)*]-1-cyano-2-phenylethyl)-4-methylpentanamide (**127**)

Yield 58%. White solid. R_f = 0.5 (ethyl acetate/ *n*-hexane 50:50). Mp. 187 – 188 °C. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.84 (d, J = 8.0 Hz, 1H), 8.02 (t, J = 1.6 Hz, 1H), 7.93 – 7.88 (m, 1H), 7.80 – 7.74 (m, 1H), 7.71 – 7.68 (m, 2H), 7.64 (t, 3J = 8.0 Hz, 1H), 7.54 – 7.50 (m, 2H), 7.45 – 7.43 (m, 1H), 7.29 – 7.19 (m, 5H), 4.82 – 4.77 (m, 1H), 3.68 (q, J = 6.0 Hz, 1H), 3.04 (dd, J = 13.2, 6.4 Hz, 1H), 2.85 (dd, J = 13.6, 9.2 Hz, 1H), 1.25 – 1.23 (m, 1H), 1.12 – 1.05 (m, 1H), 0.94 – 1.00 (m, 1H), 0.67 (d, J = 6.4 Hz, 3H), 0.58 (d, J = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 170.91, 141.75, 140.98, 130.53, 129.66, 129.32, 128.24, 128.11, 127.0, 125.6, 125.49, 125.0, 124.61, 118.77, 54.39, 41.35, 40.96, 37.43, 23.56, 22.57, 21.32. ESI-MS (+) Calc. for $[\text{C}_{27}\text{H}_{29}\text{N}_3\text{O}_3\text{S}]$ 475.6,

found: 476.2 $[M+H]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 22.26. Purity: 95.2%.

(S)-*N*-((*S*)-1-cyano-2-phenylethyl)-2-(4'-fluoro-[1,1'-biphenyl]-3-ylsulfonamido)-4-methylpentanamide (**128**)

Yield 53%. White solid. R_f = 0.3 (ethyl acetate/ *n*-hexane 50:50). Mp. 193 – 194 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.84 (d, J = 8.0 Hz, 1H, D₂O exch.), 8.10 (br d, J = 7.5 Hz, 1H, D₂O exch.), 7.99 (t, J = 2.0 Hz, 1H), 7.87 (m, 1H), 7.78 – 7.72 (m, 3H), 7.63 (t, J = 7.5 Hz, 1H), 7.39 – 7.35 (m, 2H), 7.27 – 7.19 (m, 5H), 4.81 – 4.77 (m, 1H), 3.68 (m, 1H), 3.03 (dd, 3J = 13.5, 6.5 Hz, 1H), 2.84 (dd, 3J = 13.5, 9.0 Hz, 1H), 1.26 – 1.24 (m, 1H), 1.10 – 1.04 (m, 1H), 0.93 – 0.99 (m, 1H), 0.66 (d, 3J = 7.0 Hz, 3H), 0.58 (d, 3J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ . 170.96, 162.26 (d, 2J = 243.9 Hz), 141.71, 139.88, 135.34 (d, 4J = 2.0 Hz), 130.50, 129.61, 129.37, 128.9 (d, 3J = 8.2 Hz), 128.25, 127.0, 125.11, 124.58, 118.77, 115.91 (d, J = 21.5 Hz), 54.36, 41.33, 37.40, 23.59, 22.51, 21.32. ESI-MS (+) Calc. for $[C_{27}H_{28}FN_3O_3S]$ 493.59, found: 516.1 $[M+Na]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 34.10. Purity > 99.9%.

(S)-2-([1,1'-biphenyl]-4-ylsulfonamido)-*N*-((*S*)-1-cyano-2-phenylethyl)-4-methylpentanamide (**129**)

Yield 56%. White solid. R_f = 0.7 (ethyl acetate/ *n*-hexane 50:50). Mp. 181 – 182 °C. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.90 (d, J = 8.1 Hz, 1H), 8.07 (d, J = 8.5 Hz, 1H), 7.72 – 7.59 (m, 4H), 7.52 – 7.34 (m, 4H), 7.28 – 7.18 (m, 6H), 4.82 – 4.75 (m, 1H), 3.71 (dd, J = 9.0, 5.8 Hz, 1H), 3.09 – 3.03 (m, 1H), 2.89 (dd, J = 13.5, 9.3 Hz, 1H), 1.33 – 1.25 (m, 1H), 1.18 – 1.11 (m, 1H), 1.01 – 0.96 (m, 1H), 0.70 (d, J = 6.6 Hz, 2H), 0.62 (d, J = 6.5 Hz, 3H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 171.26, 147.67, 144.12, 140.50, 140.25, 140.09, 139.02, 135.67, 129.73, 129.41, 129.30, 128.75, 128.60, 127.90, 127.55, 127.36, 127.08, 126.51, 126.37, 119.23, 54.77, 41.74, 41.19, 37.80, 23.95, 22.92, 21.72. ESI-MS (+) Calc. for $[\text{C}_{27}\text{H}_{29}\text{N}_3\text{O}_3\text{S}]$ 475.6, found: 498.1 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 35:65): t_R (min) = 7.10. Purity > 99.9%.

(S)-2-(3-chlorophenylsulfonamido)-*N*-(1-cyanocyclopropyl)-4-methylpentanamide (**130**)

Yield 81%. White solid. R_f = 0.7 (ethyl acetate/ *n*-hexane 70:30). Mp. 166 – 168 °C. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.87 (d, J = 8.1 Hz, 1H), 8.53 (d, J = 8.5 Hz, 1H), 7.70 – 7.62 (m, 1H), 7.59 (t, J = 2.0 Hz, 1H), 7.55 (dt, J = 8.0, 1.5 Hz, 1H),), 7.49 (t, J = 8.0 Hz, 1H), 3.93 – 3.89 (m, 1H), 1.61 – 1.54 (m, 1H), 1.49 – 1.46 (m, 1H), 1.45 – 1.41 (m, 1H), 1.35 – 1.32 (m, 1H), 1.11 – 1.07 (m, 1H), 0.87 (d, 3J = 8.5 Hz, 2H), 0.86 (d, 3J = 8.5 Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 171.68, 143.35, 136.74, 133.94, 132.68,

131.42, 129.64, 127.07, 78.47, 52.83, 40.75, 28.60, 24.65, 23.31, 21.94, 20.18. ESI-MS (+) Calc. for $[C_{16}H_{20}ClN_3O_3S]$ 369.86, found: 392.1 $[M+Na]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 24.15. Purity= 96.6%.

(S)-2-([1,1'-biphenyl]-3-ylsulfonamido)-*N*-(1-cyanocyclopropyl)-4-methylpentanamide
(131)

Yield 51%. White solid. R_f = 0.4 (ethyl acetate/ *n*-hexane 70:30). Mp. 163 – 165 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.88 (s, 1H), 8.17 (d, J = 8.4 Hz, 1H), 7.98 (t, J = 1.7 Hz, 1H), 7.93 – 7.89 (m, 1H), 7.74 – 7.68 (m, 3H), 7.64 (t, J = 7.7 Hz, 1H), 7.57 – 7.50 (m, 2H), 7.48 – 7.41 (m, 1H), 3.67 (dd, J = 14.3, 8.6 Hz, 1H), 1.61 – 1.54 (m, 1H), 1.49 – 1.46 (m, 1H), 1.45 – 1.43 (m, 1H), 1.33 – 1.31 (m, 1H), 1.12 – 1.06 (m, 1H), 0.89 (d, J = 8.5 Hz, 2H), 0.83 (d, J = 8.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.97, 141.47, 140.60, 138.45, 130.29, 129.47, 128.91, 128.01, 126.62, 125.11, 124.42, 120.04, 53.94, 41.18, 23.59, 22.42, 21.05, 19.06, 15.42, 14.75. ESI-MS (+) Calc. for $[C_{16}H_{25}N_3O_3S]$ 411.51, found: 412.1 $[M+H]^+$. HPLC (protocol A, ACN/ water 35:65): t_R (min) = 24.15. Purity= 96.2%.

(S)-2-(3-bromophenylsulfonamido)-*N*-(1-cyanocyclopropyl)-4-methylpentanamide
(132)

Yield 30%. White solid. R_f = 0.5 (ethyl acetate/ *n*-hexane 80:20). Mp. 175 – 177 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.90 (s, 1H), 8.31 (d, J = 8.8 Hz, 1H), 7.87 (t, J = 1.6 Hz,

1H), 7.83 (ddd, $J = 7.9, 2.0, 0.8$ Hz, 1H), 7.72 (ddd, $J = 8.0, 2.0, 1.2$ Hz, 1H), 7.52 (t, $J = 8.0$ Hz, 1H), 3.71 – 3.67 (m, 1H), 1.60 – 1.49 (m, 1H), 1.43 – 1.24 (m, 4H), 0.95 – 0.91 (m, 1H), 0.84 (d, $J = 6.4$ Hz, 3H), 0.75 (d, $J = 6.4$ Hz, 3H), 0.56 – 0.61 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.2, 143.1, 135.2, 131.3, 128.9, 125.4, 121.8, 120.2, 54.1, 41.3, 23.8, 22.6, 21.2, 19.3, 15.7, 15.1. ESI-MS (+) Calc. for $[\text{C}_{16}\text{H}_{20}\text{BrN}_3\text{O}_3\text{S}]$ 414.31, found: 437.9 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 60:40): t_R (min) = 8.33. Purity= 97.5%.

(S)-2-([1,1'-biphenyl]-4-ylsulfonamido)-*N*-(1-cyanocyclopropyl)-4-methylpentanamide
(133)

Yield 54%. White solid. $R_f = 0.7$ (ethyl acetate/ *n*-hexane 30:70). Mp. 185 – 183 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.90 (s, 1H), 8.12 (s, 1H), 7.93 – 7.80 (m, 4H), 7.70 (d, $J = 7.4$ Hz, 2H), 7.50 (t, $J = 7.4$ Hz, 2H), 7.42 (t, $J = 7.3$ Hz, 1H), 3.71 – 3.66 (m, 1H), 1.60 – 1.54 (m, 1H), 0.90 – 0.80 (m, 4H), 0.95 – 0.91 (m, 1H), 0.74 (d, $J = 6.5$ Hz, 3H), 0.71 (d, $J = 6.5$ Hz, 3H), 0.58 – 0.51 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.50, 144.17, 139.99, 138.85, 129.32, 128.95, 128.65, 127.41, 127.35, 127.25, 120.54, 54.42, 41.62, 24.05, 22.90, 21.49, 19.56, 15.77, 15.36. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_3\text{S}]$ 414.51, found: 434.1 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 28.57. Purity= 97.6%.

(4'-Fluorobiphenyl-4-yl)sulfonyl-leucyl-aminocyclopropane-nitrile (**134**)

Yield 53%. White solid. R_f = 0.7 (ethyl acetate/ *n*-hexane 30:70). Mp. 199 – 200 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.88 (s, 1H), 8.14 (s, 1H), 7.92 – 7.88 (m, 4H), 7.39 – 7.30 (m, 4H), 3.71 – 3.66 (m, 1H), 1.60 – 1.54 (m, 1H), 0.90 – 0.80 (m, 4H), 0.95 – 0.91 (m, 1H), 0.74 (d, J = 6.5 Hz, 3H), 0.71 (d, J = 6.5 Hz, 3H), 0.58 – 0.51 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 172.01, 161.56 (d, J = 243.9 Hz), 142.68, 136.42, 133.22, 131.32, 131.09, 128.95, 127.48, 127.56, 119.87, 116.95 (d, $3J$ = 21.5 Hz), 116.31 (d, J = 21.5 Hz), 54.88, 41.73, 24.25, 22.91, 21.59, 19.52, 15.71, 15.37. ESI-MS (+) Calc. for $[\text{C}_{22}\text{H}_{24}\text{FN}_3\text{O}_3\text{S}]$ 429.5, found: 452.4 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 10.32. Purity = 99.6%.

(*S*)-2-(2-chlorophenylsulfonylamido)-*N*-(1-cyanocyclopropyl)-4-methylpentanamide (**135**)

Yield 60%. White solid. Mp. 170 – 172 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.79 (s, 1H), 8.17 (d, J = 5.3 Hz, 1H), 7.91 (dt, J = 8.1, 0.7 Hz, 1H), 7.62 – 7.58 (m, 2H), 7.51 – 7.51 (m, 1H), 3.61 – 3.66 (m, 1H), 1.56 – 1.64 (m, 1H), 1.48 (ddd, J = 13.6, 9.9, 5.1 Hz, 1H), 1.32 – 1.40 (m, 2H), 1.25 (ddd, J = 13.8, 8.8, 5.1 Hz, 1H), 0.88 – 0.84 (m, 1H), 0.81 (d, J = 6.7 Hz, 3H), 0.68 (d, J = 6.6 Hz, 3H), 0.66 – 0.62 (m, 1H). ^{13}C NMR (125 MHz, DMSO) δ 172.43, 138.23, 134.14, 131.81, 131.22, 130.48, 127.48, 120.48, 54.55, 41.14, 23.93, 22.91, 21.13, 19.56, 15.80, 15.38. ESI-MS (+) Calc. for $[\text{C}_{16}\text{H}_{20}\text{ClN}_3\text{O}_3\text{S}]$ 369.8, found: 392.7 $[\text{M}+\text{Na}]^+$. HPLC (protocol C): t_R (min) = 9.19. Purity > 99.9%.

(S)-2-(3-chlorophenylsulfonamido)-*N*-(1-cyanocyclopropyl)-3-phenylpropanamide
(136)

Yield 66%. White solid. Mp. 193 – 194 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.88 (s, 1H), 8.54 (s, 1H), 7.62 (ddd, $J = 7.0, 2.5, 1.0$ Hz, 1H), 7.59 (t, $J = 2.0$ Hz, 1H), 7.55 (dt, $J = 8.0, 1.5$ Hz, 1H), 7.49 (t, $J = 8.0$ Hz, 1H), 7.23 – 7.16 (m, 3H), 7.12 – 7.00 (m, 2H), 3.89 – 3.87 (m, 1H), 2.82 (dd, $J = 13.5, 6.5$ Hz, 1H), 2.71 (dd, $J = 13.5, 7.0$ Hz, 1H), 0.80 – 0.77 (m, 2H), 0.58 – 0.53 (m, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 171.68, 143.35, 136.74, 133.94, 132.68, 131.42, 129.64, 128.51, 127.07, 126.44, 125.31, 120.50, 57.63, 38.90, 19.66, 16.03, 15.72. ESI-MS (+) Calc. for $[\text{C}_{19}\text{H}_{18}\text{ClN}_3\text{O}_3\text{S}]$ 403.8, found: 426.4 $[\text{M}+\text{Na}]^+$. HPLC (protocol A, ACN/ water 50:50): t_R (min) = 11.21 Purity= 99%.

(S)-2-([1,1'-biphenyl]-3-ylsulfonamido)-*N*-(1-cyanocyclopropyl)-3-phenylpropanamide
(137)

Yield 71%. White solid. Mp. 168 – 170 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.78 (s, 1H), 8.38 – 8.32 (m, 1H), 7.90 (t, $J = 1.7$ Hz, 1H), 7.85 (dt, $J = 7.6, 1.4$ Hz, 1H), 7.68 – 7.65 (m, 2H), 7.62 (dt, $J = 7.8, 1.3$ Hz, 1H), 7.56 (t, $J = 7.7$ Hz, 1H), 7.54 – 7.49 (m, 2H), 7.45 – 7.41 (m, 2H), 7.19 – 7.15 (m, 1H), 7.14 – 7.10 (m, 1H), 7.10 – 7.07 (m, 2H), 3.92 – 3.86 (m, 1H), 2.82 (dd, $J = 13.5, 7.1$ Hz, 1H), 2.70 (dd, $J = 13.5, 8.0$ Hz, 1H), 1.25 – 1.22 (m, 2H), 0.87 – 0.77 (m, 1H), 0.65 – 0.58 (m, 1H). ^{13}C NMR (125 MHz, DMSO) δ 171.44, 141.89, 140.98, 138.87, 136.48, 130.65, 129.81, 129.34, 129.26, 128.35, 128.22, 127.05, 126.71, 125.33, 124.70, 120.20, 57.29, 38.76, 19.30, 15.74,

15.26. ESI-MS (+) Calc. for $[C_{25}H_{23}N_3O_3S]$ 445.5, found: 468.3 $[M+Na]^+$. HPLC (protocol C): t_R (min) = 10.75. Purity= 98.5%.

(S)-2-([1,1'-biphenyl]-4-ylsulfonamido)-*N*-(1-cyanocyclopropyl)-3-phenylpropanamide
(138)

Yield 76%. White solid. Mp 186 – 188 °C. 1H NMR (500 MHz, $DMSO-d_6$) δ 8.84 – 8.78 (m, 1H), 8.34 – 8.26 (m, 1H), 7.76 – 7.70 (m, 2H), 7.71 – 7.64 (m, 4H), 7.52 – 7.47 (m, 2H), 7.42 (tt, J = 7.3, 1.6 Hz, 1H), 7.24 – 7.12 (m, 3H), 7.14 – 7.08 (m, 2H), 3.88 (app. q, J = 7.2 Hz, 1H), 2.82 (dd, J = 13.6, 6.8 Hz, 1H), 2.72 (dd, J = 13.6, 8.2 Hz, 1H), 1.34–1.20 (m, 2H), 0.78 – 0.72 (m, 1H), 0.55 – 0.48 (m, 1H). ^{13}C NMR (125 MHz, $DMSO-d_6$) δ 171.55, 143.97, 139.97, 138.85, 136.59, 129.42, 129.26, 128.56, 128.26, 127.28, 127.20, 127.14, 126.68, 120.31, 57.34, 38.69, 19.37, 15.67, 15.42. HRMS (+) Calc. for $[C_{25}H_{23}N_3O_3S]^+$ 446.1533, found: 446.1532 $[M+H]^+$. HPLC (protocol C): t_R (min) = 10.75 Purity= 98.2%.

(S)-2-(2-chlorophenylsulfonamido)-*N*-(1-cyanocyclopropyl)-3-phenylpropanamide
(139)

Yield 74%. Mp 130 – 132 °C. 1H NMR (500 MHz, $DMSO-d_6$) δ 8.77 (s, 1H), 8.34 (s, 1H), 7.72 (dd, J = 7.9, 1.5 Hz, 1H), 7.56 – 7.49 (m, 2H), 7.40 – 7.35 (m, 1H), 7.21 – 7.11 (m, 5H), 3.87 (t, J = 7.3 Hz, 1H), 2.85 (dd, J = 13.6, 6.5 Hz, 1H), 2.79 (dd, J = 13.6, 8.6 Hz, 1H), 1.36 – 1.30 (m, 2H), 0.77 – 0.72 (m, 1H), 0.60 – 0.55 (m, 1H). ^{13}C NMR (125 MHz, $DMSO$) δ 171.49, 138.27, 136.60, 133.90, 131.79, 131.03, 130.03,

129.36, 128.20, 127.32, 126.68, 120.30, 57.62, 38.30, 19.40, 15.74, 15.45. ESI-MS (+)
Calc. for $[C_{19}H_{18}ClN_3O_3S]$ 403.9, found: 404.9 $[M+H]^+$. HPLC (protocol C): t_R (min) =
9.29. Purity= 97.3%.

8.2. Enzyme inhibition studies

Recombinant cruzain was expressed and activated by M. Sc. Fabiana Rosini as elsewhere reported (CIANNI et al., 2018). CatL was purchased by Enzo Life Science®.

8.2.1. Determination of the Michaelis-Menten constants for cruzain and CatL

The enzyme kinetics assays to determine the K_M were done at 25 °C using a 96-well black microplate (Corning®) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The enzymes were activated with assay buffer 100 mM sodium acetate pH 5.5, 300 mM of NaCl and 5 mM of EDTA, containing 7 mM DTT, 0.014% of Triton X-100, and was then incubated in an ice bath for 20 min. The concentrations used for the enzymes were 0.15 nM and 1.9 nM for cruzain, and Cat-L respectively. The reaction rate was followed for 5 min in the fluorimeter, in which the rate of hydrolysis of the substrate Z-Phe-Arg-AMC was monitored. A final concentration of DMSO of 5% (10 µL) in each well was used. The assays were performed in triplicates. Eight different substrate concentrations, prepared with a 0.5 dilution factor, were used.

$$K_M = 1.8 \pm 0.2 \text{ (Cz)}$$

$$K_M = 3.3 \pm 0.2 \text{ (CatL)}$$

8.2.2. Determination of the inhibition constant (K_i) for cruzain and CatL

Recombinant cruzain and CatL were assayed by fluorometric assay (Biotek® Synergy™ HT) monitoring the hydrolysis rate of the fluorogenic substrate Z-Phe-Arg-7-amido-4-methylcoumarin (Z-FR-AMC, Sigma-Aldrich) with fluorescence emission at 460 nm (excitation at 355 nm) and at 37°C. The reactions were followed over 5 min for fast-binding and irreversible inhibitors and over 30 min for compounds displaying slow-binding behavior, respectively. Enzyme kinetic assays were carried out in 200 µL of a

solution containing 100 mM acetate buffer pH 5.5, 300 mM NaCl, 5 mM DTT (dithiothreitol), 5% v/v DMSO (dimethyl sulfoxide), 0.01% v/v Triton X-100 and 0.15 nM Cruzain or 1.9 nM of CatL, using Corning® 96-well black flat bottom microplates. The enzyme stock aliquot was rapidly thawed at 37 °C and kept on ice until activation, in which it was incubated for 20 min in the assay buffer (100 mM acetate pH 5.5 and 5 mM DTT) followed by additional 2 min with inhibitors before the reaction to be started by the addition of the substrate (final concentration equal to K_M of the enzyme). Visual inspection and a pre-reading of plate wells were performed to check respectively for possible precipitation and background fluorescence. None of the compounds displayed a significant fluorescence signal around 460 nm, the emission wavelength used to monitor reaction kinetics. Thus, potential inner-filter effects did not have to be taken into account in our experiments. Analysis and manipulation of the data were performed by Sigma Plot 10.

8.2.3. Kinetic parameters determination of fast-binding inhibitors

Each experiment was performed in triplicate for each compound. Initial velocities of the substrate hydrolysis under first-order reaction were calculated by Gen5™ Biotek® software. The apparent inhibition constant K_i' was determined by non-linear regression using equation $v_s = V_o/(1+[I]/K_i')$, where v_s is the steady-state rate, V_o 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. A control measurement in presence of the covalent reversible fast-binding inhibitor **Neq0409** was done for each setup plate. All inhibitors were evaluated at seven different concentrations that were chosen based on a previous screening (percent inhibition) at 1.7 μ M substrate ($\sim K_M$) concentration (LÖSER et al., 2008).

Figure 31. Reversible fast-binding behavior of **Neq0710**.

Source: Own author.

As a general example the kinetic assays for **Neq0710** is reported here. Monitoring cruzain-catalyzed hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin in the presence of increasing concentrations of compound **Neq0710** (starting from 10 μM, 7 concentrations, dilution of 0.5; Figure 32). The reaction (pH 5.5, 300 mM NaCl, 5 mM DTT (dithiothreitol), 5% v/v DMSO (dimethyl sulfoxide), 0.01% v/v Triton X-100 and 0.15 nM Cruzain) was initiated by addition of the substrate (1.7 μM) after incubating the inhibitor with the enzyme for 2 min. Fluorescence emission at 440 nm was measured after excitation at 360 nm. Fluorescence units (FU) were corrected for background fluorescence. The rate of hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin was plotted *versus* concentrations of **Neq0710**. Non-linear regression gave an apparent inhibition constant $K'_i = (1+[S]/K_M)K_i = 72.2 \pm 3.6$ nM. The same method was applied for the others nitrile based compounds as well for CatL.

8.2.4. Kinetic parameters determination of slow-binding inhibitors

To determine the kinetic parameters associated with slow binding inhibition of cruzain, progress curves in the presence of azadipeptide nitriles **Neq0676**, **Neq0677**, **Neq0689** and **Neq0690** were obtained in the presence for eleven different concentrations of

inhibitor using fixed Z-FR-AMC substrate concentration of 30 μ M. Each progress curve was analyzed by non-linear regression using slow-binding Equation 1 described by Morrison and Walsh (TOONE, 2008):

$$[P] = v_s t + \frac{(v_i - v_s)(1 - e^{-k_{obs}t})}{k_{obs}} + d \quad (\text{equation 1})$$

Whereas [P] is the product concentration, 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. v_s and v_0 values were plotted versus the inhibitor concentration [I], according to equation 2 to obtain K'_i :

$$v_s = \frac{v_0}{1 + [I]/K'_i} \quad (\text{equation 2})$$

The K_i value was calculated from equation 3:

$$K_i = \frac{K'_i}{1 + [S]/K_M} \quad (\text{equation 3})$$

The apparent second-order rate constant k'_{on} was obtained by linear regression according to equation 4:

$$k_{obs} = k'_{on}[I] + k_{off} \quad (\text{equation 4})$$

The true rate constant k_{on} was calculated by correction of k'_{on} according to equation 5:

$$k_{on} = k'_{on}(1 + [S]/K_M) \quad (\text{equation 5})$$

The first-order rate constant k_{off} for the dissociation of the enzyme-inhibitor complex was calculated according to equation 6:

$$k_{off} = k_{on}K_i \quad (\text{equation 6})$$

Figure 32. Slow-binding behavior of azadipeptide nitrile (**Neq0677**).

Up: Measuring RFU (sec^{-1}) Vs time (sec) for **Neq0677** for 3600sec, for eleven different concentrations. Down: Slow-binding behavior of azadipeptide nitriles. Source: Retrieved from SILVA, G. D. et al. A comparative study of warheads for design of cysteine protease inhibitors. **Bioorganic & Medicinal Chemistry Letters**, v. 27, p. 5031–5035, oct. 2017.

As a general example the kinetic assays for **Neq0677** are reported here. It was monitoring (Figure 7a and 7b) the cruzain-catalyzed hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin (30 μM) in the presence of increasing concentrations of compound **Neq0677** (eleven concentrations, from 500nM, dilution of 0.7). The reaction (pH 5.5, 300 mM NaCl, 5 mM DTT (dithiothreitol), 5% v/v DMSO (dimethyl sulfoxide), 0.01% v/v Triton X-100 and 0.15 nM Cruzain) was initiated by addition of the enzyme.

Fluorescence emission at 440 nm was measured after excitation at 360 nm. Fluorescence units (FU) were corrected for background fluorescence. Plot of the rates of hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin *versus* concentrations of **Neq0677**. Non-linear regression gave an apparent inhibition constant $K'_i = (1+[S]/K_M)K_i$. The inset is a plot of the first-order rate constants k_{obs} *versus* concentrations of **Neq0677**. Linear regression gave a second-order rate constant $k_{on}' = k_{on} / (1+([S]/K_M))$.

8.2.5. Assessment of reversibility mode of binding

The reversibility of compound **Neq0677**, **Neq0689** and **Neq0690** to Cz and CatL was tested by measuring the recovery of enzyme activity after a rapid and extensive dilution of the enzyme-inhibitor complex. The inhibitor was incubated at a concentration 10 times greater than its K_i value with 100 times the concentration of the enzyme required for enzyme activity assay for 30 min. The mixture was then diluted by a factor of 100 in the reaction buffer, containing the Z-Phe-Arg-AMC substrate at the final concentration of 7 μ M as controls compound **Neq0570** (reversible) and E-64 inhibitor (irreversible) were used. Recovery of enzyme activity after dilution of the inhibitor Cz (or CatL) complex suggests a reversible inhibition mechanism for **Neq0690**.

Figure 33. Reversibility plot of compound **Neq0690** with Cz.

E-64 (irreversible, black line); **Neq0690** (reversible, red line); **Neq0570** (reversible, green line). Source: Retrieved from SILVA, G. D. et al. A comparative study of warheads for design of cysteine protease inhibitors. **Bioorganic & Medicinal Chemistry Letters**, v. 27, p. 5031–5035, oct. 2017.

9. FINAL CONCLUSIONS AND OUTLOOK

An extensive analysis of structure-activity relationships (SARs) has been performed on nitrile-based cysteine protease inhibitors. Some of the crucial reasons for inhibition of cysteine proteases in infectious diseases (Chagas and Leishmaniasis) and in humans (for instance, Chagas heart diseases) have been adequately mapped. By applying a knowledge-based design approach, we have designed and synthesized a new series of over 40 dipeptidyl nitrile inhibitors of Cz which has led to leverage the S3/P3 interactions, understand the role of the cyclopropane moiety in the bimolecular recognition process and map the S1/S1' to increase affinity and selectivity around the studied PCs. Following the engineering of the hereof molecular modifications, a new generation of dipeptidyl nitriles was created as high-affinity Cz inhibitors. Inhibitors **Neq0887**, **Neq0938** and **Neq0940** showed trypanocidal activity, EC₅₀ (*T. cruzi*) of about 4 μ M in a low micromolar range, with low effect on host cell cytotoxicity (SI > 20). Hence, they may be considered as new candidates for *in vivo* studies against the acute form of Chagas disease. Besides that, combination of P3 and P2 groups led to the first dipeptidyl nitrile synthesized in our group to have a difference in affinity between Cz and CatL over one log unit in favor of the protozoan CP. In addition, kinetic evaluation on a panel of human cathepsins resulted in three new inhibitors at one-digit CatK nanomolar concentrations (i.e. **Neq0955** and **Neq0958**) and eight inhibitors at one-digit CatL nanomolar concentrations (i.e. **Neq0953** and **Neq0954**).

In addition, we have performed an extensive SAR (over 25 compounds) for non-peptidic nitrile based inhibitors reporting one-digit nanomolar Cz inhibition (**Neq0643**, **Neq0659** and **Neq0820**) with good selectivity over CatL. This work likewise elucidates the influence of the stereochemistry of the trifluoroethylamine group in P2/P3 and displays a new way to represent a nonadditive SAR. An unexpected outcome arose after the identification of cysteine protease inhibitors at low nanomolar concentrations: most of the compounds showed no trypanocidal activity. Although this event is not uncommon, it points out the difficulty and ongoing challenge to translate enzyme inhibition to trypanocidal activity. That is, despite the scientific innovation of identifying new competitive cruzain inhibitors and the discovery of promising new candidates for

Chagas disease, not all inhibitors act as trypanocidal agents and therefore further research is warranted.

Similarly, we have evaluated the sulfonamide bond in P3/P2 as bioisosteres of the amide bond by performing the design, synthesis and biological study of a new set of reversible inhibitors. The flat SAR for Cz suggests a new mode of binding that needs to be elucidated in future studies. Yet, out of the same set of compounds, the inhibitor **Neq0830** exhibits a strong CatS affinity (pK_i 8.4) with selectivity of more than one log unit over other mammalian CPs.

Alternative warhead effects to nitrile have been explored for Cz inhibition. Biochemical assays present azadipeptide nitriles with two magnitude orders higher Cz affinity than the corresponding dipeptide nitriles. Furthermore, azadipeptide nitriles display low micromolar potency as *T. cruzi* killers but with no selectivity in respect to the host cell. Nevertheless, the azadipeptide nitrile compound (**Neq0690**) was used for the generation of the first high-resolution x-Ray crystal structure of *Leishmania mexicana* cysteine protease B (LmCPB).

In perspective, the synthesis of dipeptidyl nitriles should be used to map new subsites and to endeavor non-natural amino acids. In particular, the structure of **Neq0940**, **Neq0938** and **Neq0877** should be optimized to improve their pharmacokinetics and pharmacodynamics profiles. Also, research should be focused on new possible bioisosteres of the carbonyl group (*i.e.* oxetane, currently being pursued in our research group) of the amide bond in P3/P2 position to gain metabolic stability without loss of affinity towards Cz. Biological studies should try to explain the lack of trypanocidal activity for low nanomolar Cz inhibitors as for **Neq0659**, **Neq0643** and **Neq0820** to reinforce the validation of the Cz as a target or to invalidate it. Based on our results, a long-term study should focus on the discovery of new warheads for cysteine protease inhibition. The intrinsic electrophile reactivity of new warheads should be considered to yield a balanced reactive profile and to prevent nonspecific off-target while forming a desirable reversible covalent bond with the opportune cysteine protease.

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APPENDIX A

^1H NMR and ^{13}C NMR of key compounds.

Figure 34. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) of compound **4** (Neq0710).

Figure 35. ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) of compound **4** (Neq0710).

Figure 36. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) of compound **6** (Neq0712).

Figure 37. ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) of compound **6** (Neq0712).

Figure 38. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) of compound **7** (Neq0717).

Figure 39. ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) of compound **7** (Neq0717).

Figure 40. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) of compound **9** (Neq0809).

Figure 41. ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) of compound **9** (Neq0809).

Figure 42. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) of compound **10** (Neq0804).

Figure 43. ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) of compound **10** (Neq0810).

Figure 44. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) of compound **24** (**Neq0865**).

Figure 45. ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) of compound **24** (Neq0865).

Figure 46. ^1H NMR (200 MHz, CDCl_3) of compound **29** (Neq0956). **Figure 47.** ^{13}C NMR (50 MHz, CDCl_3) of compound **29** (Neq0956).

Figure 48. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of compound **31** (Neq0775).

Figure 49. ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) of compound **31** (Neq0775).

Figure 50. ^1H NMR (200 MHz, CD_3OD) of compound **36** (Neq0957). **Figure 51.** ^{13}C NMR (50 MHz, CD_3OD) of compound **36** (Neq0957).

Figure 52. ^1H NMR (400 MHz, CDCl_3) of compound **67** (Neq0937). **Figure 53.** ^{13}C NMR (50 MHz, CDCl_3) of compound **29** (Neq0956).

Figure 54. ^1H NMR (200 MHz, CD_3OD) of compound **71** (Neq0953). **Figure 55.** ^{13}C NMR (50 MHz, CD_3OD) of compound **71** (Neq0953).

Figure 56. ^1H NMR (400 MHz, CD_3OD) of compound **72** (Neq0954). **Figure 57.** ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) of compound **72** (Neq0954).

Figure 58. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) of compound **75** (Neq0922).

Figure 59. ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) of compound **75** (Neq0922).

Figure 60. ^1H NMR (400 MHz, CDCl_3) of compound **77** (Neq0877). **Figure 61.** ^{13}C NMR (100 MHz, CDCl_3) of compound **77** (Neq0877).

Figure 62. ^1H NMR (400 MHz, CDCl_3) of compound **84** (Neq0942). **Figure 63.** ^{13}C NMR (100 MHz, CDCl_3) of compound **84** (Neq0942).

Figure 64. ^1H NMR (400 MHz, CD_3OD) of compound **85** (**Neq0944**).

Figure 65. ^{13}C NMR (100 MHz, CDCl_3) of compound **85** (Neq0944).

Figure 66. ^1H NMR (400 MHz, CD_3OD) of compound **86** (Neq0948). **Figure 67.** ^{13}C NMR (100 MHz, CDCl_3) of compound **86** (Neq0948).

Figure 68. ^1H NMR (400 MHz, CDCl_3) of compound **111** (Neq0670). **Figure 69.** ^{13}C NMR (100 MHz, CDCl_3) of compound **111** (Neq0670).

Figure 70. ^1H NMR (400 MHz, CDCl_3) of compound **112** (Neq0643).

Figure 71. ^{13}C NMR (100 MHz, CDCl_3) of compound **112** (Neq0643).

Figure 72. ^1H NMR (400 MHz, CDCl_3) of compound **113** (Neq0658).

Figure 73. ^{13}C NMR (100 MHz, CDCl_3) of compound **113** (Neq0658).

Figure 74. ^1H NMR (400 MHz, CDCl_3) of compound **121** (Neq0820). **Figure 75.** ^{13}C NMR (100 MHz, CDCl_3) of compound **121** (Neq0820).

Figure 76. ^{19}F NMR (376 MHz, CDCl_3) of compound **121** (Neq0820).

Figure 77. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) of compound **124** (Neq0819).

Figure 78. ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) of compound **124** (Neq0819).

Figure 79. ^1H NMR (400 MHz DMSO- d_6) of compound **125** (Neq0781).

Figure 80. ^{13}C NMR (100 MHz, DMSO- d_6) of compound **125** (Neq0781).

Figure 81. ^1H NMR (500 MHz $\text{DMSO-}d_6$) of compound **126** (Neq0782).

Figure 82. ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$) of compound **126** (Neq0782).

Figure 83. ^1H NMR (400 MHz DMSO- d_6) of compound **127** (Neq0831).

Figure 84. ^{13}C NMR (100 MHz, DMSO- d_6) of compound **127** (Neq0831).

Figure 85. ^1H NMR (400 MHz DMSO- d_6) of compound **131** (Neq0830).

Figure 86. ^{13}C NMR (100 MHz, DMSO- d_6) of compound **131** (Neq0830).

Figure 87. ^1H NMR (400 MHz DMSO- d_6) of compound **134** (Neq0838). **Figure 88.** ^{13}C NMR (100 MHz, DMSO- d_6) of compound **134** (Neq0838).

APPENDIX B

HPLC analysis to evaluate the absence of racemization in the synthetic route.

Figure 89. HPLC analysis of *R*-99.

1 PDA Multi 1 / 206nm 4nm

Chiralpak® IC-5 (5 μ m), 250 x 4.6 mm, ACN/ water: 30:70, 206 nm; stop time 40 min, flow 0.5 ml/min.

Figure 90: HPLC analysis of *S*-99.

1 PDA Multi 1 / 206nm 4nm

Chiralpak® IC-5 (5 μ m), 250 x 4.6 mm, ACN/ water: 30:70, 206 nm; stop time 40 min, flow 0.5 ml/min.

Figure 91. HPLC analysis of a mixture of *R*-99 and *S*-99.

Chiralpak® IC-5 (5 μ m), 250 x 4.6 mm, ACN/ water: 30:70, 206 nm; stop time 40 min, flow 0.5 ml/min.

Figure 92. HPLC analysis of **67 (Neq0937)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 30 min, stop time 40 min, flow 0.5 ml/min.

Figure 93. HPLC analysis of **68 (Neq0866.1)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 30 min, stop time 40 min, flow 0.5 ml/min.

Figure 94. HPLC analysis for a mixture of **67 (Neq0937)** and **68 (Neq0866.1)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 30 min, stop time 40 min, flow 0.5 ml/min.

Figure 95. HPLC analysis of **73 (Neq0921)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 30 min, stop time 40 min, flow 0.5 ml/min.

Figure 96. HPLC analysis of **74 (Neq0938)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 40 min, stop time 30 min, flow 0.5 ml/min.

Figure 97. HPLC analysis for a mixture of **73 (Neq0921)** and **74 (Neq0938)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 30 min, stop time 40 min, flow 0.5 ml/min.

Figure 98. HPLC analysis of **82 (Neq0941)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 40 min, stop time 30 min, flow 0.5 ml/min.

Figure 99. HPLC analysis of **83 (Neq0943)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 40 min, stop time 30 min, flow 0.5 ml/min.

Figure 100. HPLC analysis for a mixture of **82 (Neq0941)** and **83 (Neq0943)** with Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 μ m).

Elution was carried out with the following gradient: water (solvent A), ACN (solvent B), 5% B to 100% B in 30 min, stop time 40 min, flow 0.5 ml/min.