Published in final edited form as:

ChemMedChem. 2010 October 4; 5(10): 1734–1748. doi:10.1002/cmdc.201000265.

α -Ketoheterocycles as inhibitors of *Leishmania mexicana* cysteine protease CPB

Dr. Koen Steert^[a], Dr. Maya Berg^[a], Prof. Dr. Jeremy C. Mottram^[b], Dr. Gareth D. Westrop^[c], Prof. Dr. Graham H. Coombs^[c], Prof. Dr. Paul Cos^[d], Prof. Dr. Louis Maes^[d], Dr. Jurgen Joossens^[a], Prof. Dr. Pieter Van der Veken^[a], Prof. Dr. Achiel Haemers^[a], and Prof. Dr. Koen Augustyns^{*,[a]}

^[a]Department of Medicinal Chemistry, University of Antwerp Universiteitsplein 1, 2610 Antwerp (Belgium)

^[b]Wellcome Centre for Molecular Parasitology and Division of Infection & Immunity – Glasgow Biomedical Research Centre University of Glasgow 120 University Place, Glasgow G12 8TA (United Kingdom)

^[c]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, United Kingdom

^[d]Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp Groenenborgerlaan 171, 2020 Antwerp (Belgium)

Abstract

Cysteine proteases of the papain superfamily are present in nearly all eukaryotes and also play pivotal roles in the biology of parasites. Inhibition of cysteine proteases is emerging as an important strategy to combat parasitic diseases such as sleeping sickness, Chagas' disease and leishmaniasis. Inspired by the in vivo antiparasitic activity of the vinyl sulfone based cysteine protease inhibitors (CPIs), a series of α -ketoheterocycles **1-15** has been developed as reversible inhibitors of a recombinant *L. mexicana* cysteine protease CPB2.8. The isoxazoles **1-3** and especially the oxadiazole **15** are potent reversible inhibitors of CPB2.8, however, in vitro whole-organism screening against a panel of protozoan parasites did not fully correlate with the observed inhibition of the cysteine protease.

Keywords

cysteine proteases; inhibitors; ketoheterocycle; parasite CPB; *Trypanosoma*

Introduction

Trypanosomes are parasitic protozoa that afflict both man and animals and are responsible for several neglected diseases that remain a major health problem in developing countries. African trypanosomiasis (sleeping sickness), American trypanosomiasis (Chagas' disease) and leishmaniasis are parasitic diseases caused by kinetoplastid protozoa of the genus *Trypanosoma*; *Trypanosoma brucei*, *Trypanosoma cruzi*, and various *Leishmania* spp., respectively. Current drug therapy for the treatment of these neglected diseases mainly relies on drugs developed decades ago. Severe toxic effects combined with the emergence of drug

*Fax: (+32) 3 265 27 39 koen.augustyns@ua.ac.be.

resistant parasite strains create an urgent and continuous need for new, safe and effective drugs.^[1]

Cysteine proteases constitute a pivotal class of enzymes that play numerous roles in the biology of parasitic organisms.^[2] Identification and further characterization of cysteine protease-mediated processes in parasitic protozoa is progressing at a rapid pace.^[3] A possible strategy for combating parasitic infections is to inhibit cysteine proteases that are crucial to parasite metabolism and reproduction. Papain-like cysteine proteases have been identified in *T. cruzi* (cruzain), *T. brucei* (trypanopain, TbCatB) and different *Leishmania* spp. (CPA, CPB, CPC), and inhibition of these peptidases has led to promising results both in vitro in tissue culture models^[4,5] as in vivo.^[6,7] Our research effort has focused on CPB, a cathepsin L-like cysteine protease target present in *Leishmania mexicana* as a tandem array of 19 similar genes.^[9] CPB expression is regulated so that CPB1 and CPB2, the first two genes of the tandem array, are expressed in the infectious metacyclic stage and the remaining genes in the intracellular disease relevant amastigote stage.^[9] A recombinant form of the amastigote specific isoform CPB2.8 was used in this study.^[10] Due to their high sequence identity, the multiple isoforms are expected to be inhibited with one inhibitor.

L. mexicana mutants defective in production of CPBs have an attenuated phenotype; growth in nutrient rich culture is not affected but the mutants are less infectious for macrophages in vitro and they have reduced virulence for BALB/c mice with poor lesion growth.^[4,11] The virulence of *L. mexicana* is associated with the ability of CPB to induce IL4 and a Th2 response.^[12-14] The Δcpb mutant fails to induce IL4 and a protective Th1 response develops that facilitates wound healing. A similar phenotype was recently described for mutants overexpressing ICP, a natural inhibitor of CPB and other cathepsin L-like CPs.^[15] The reduced infectivity of *L. mexicana* Δcpb mutants for macrophages has been linked to an impairment of autophagy dependent protein turnover required for differentiation of promastigotes into infectious metacyclics.^[8,16] These studies have identified CPB as an important virulence factor and demonstrate that CPB inhibitors have therapeutic potential.

A common feature of most peptide-derived cysteine protease inhibitors is a so-called 'warhead': an electrophilic functionality, such as a carbonyl group or a Michael acceptor that is attacked by the catalytic cysteine thiolate in the active site (Figure 1). The choice of the 'warhead' -type, either irreversibly binding as for Michael acceptors^[17] or reversibly reactive, such as for ketones, is arguably often urged to the reversible types. These might be expected to possess better safety profiles with regards to their potential application as drugs for treating parasitic infections. Inspired by the in vivo anti-parasitic activity of the irreversible vinyl sulfone cruzain inhibitors, e.g. **Mu-K11777**, developed by McKerrow and co-workers, we describe here our efforts to identify structurally related compounds of the homologous papain-like cysteine protease CPB2.8 from *L. mexicana*. Contrary to the former, our inhibitors bear a reversible ketone-based 'warhead' (Figure 2), for circumventing potential toxicity risks (e.g. autoimmune drug reactions) that have been associated with irreversible inhibitor types.^[18]

Several ketone-based 'warhead' groups have been reported in literature, including: α -fluoroalkyl ketones,^[19] α -hydroxymethyl ketones,^[20] α -keto amides^[21] and α -ketoheterocycles.^[22,23] The latter, already described i.a. in inhibitors of parasite metacaspases^[24], human cathepsins, and several serine proteases^[25] have proven to be advantageous for two main reasons. First, they possess a keto-group of which the electrophilicity is 'tuneable' and depends on the nature of the heterocyclic moiety present. Second, they offer a molecular template for attaching substituents to introduce new binding interactions. Remarkably, (fused) five-ring heterocycles containing a ring nitrogen in β -position relative to the keto group in general seem to give optimal enzyme affinities. This

nitrogen has been demonstrated to provide a hydrogen-bond interaction with the highly conserved catalytic His residue in catalytic dyads and triads of both cysteine and serine proteases.^[26]

We introduced several types of heterocycles in our compounds: isoxazoles, triazoles, thiazoles, oxazoles and oxadiazoles and some of their benzo-fused analogues (Figure 3).

Owing to their high degree of homology and the similar substrate preferences of CPB2.8 and cruzain, we decided to largely retain the P₂- and P₃-overall structures of **Mu-K11777** and analogous Michael acceptor based inhibitors described by McKerrow.^[6] The P₁-position, originally occupied by homophenylalanine (hPhe, n=2 in Figure 2), is frequently replaced in our series by its naturally occurring analogue phenylalanine (Phe, n=1 in Figure 2). Both in crystal structures of cruzain and the homology model of CPB2.8 which was developed in our laboratory, the hPhe side chain was found to extend into the solvent and to not make productive interactions with the enzyme. As reported in literature the hPhe residue at this position was chosen because it increases the in vivo lifetime of the inhibitor and reduces toxicity, rather than for optimising enzyme affinity.^[20] Only for a selected series of inhibitors, the hPhe-analogue was also synthesized in order to verify whether they followed the structure-activity relationship put forward for the P₁-Phe containing compounds.

Results and Discussion

Chemistry

Several synthetic strategies for the synthesis of α -ketoheterocycle containing peptide derived inhibitors are described in literature. Important methods are addition of metalated heterocycles to aldehydes, Weinreb amides or esters and ring construction from α -cyanohydrins and imidates. Our different synthetic strategies towards compounds **1-15** can be categorized into 3 distinct approaches: i) 1,3-dipolar cycloaddition, ii) direct metalation of the heterocycle or iii) heterocycle formation via a cyanohydrin intermediate.

The synthesis of isoxazoles **1-5** is started with commercially available Boc-protected phenylalanine (Scheme 1), that was converted into Boc-phenylalaninal (Boc-Phe-H) **18** in two steps using *N,O*-dimethylhydroxylamine followed by reduction with LiAlH₄.^[27] The nitroalcohol **20** was synthesized by a Henry reaction between Boc-Phe-H **18** and nitromethane using TBAF as the catalyst.^[28] Prior to cycloaddition, the nitroalcohol **20** was temporarily protected. TMS-protected nitroalcohol **22** was converted, according to Mukaiyama's method,^[29] into the nitrile oxide 1,3-dipole *in situ* and is immediately reacted further by the addition of an appropriate alkyne. Removal of the TMS group was done with acetic acid/water (80:20) or citric acid in methanol. Boc-deprotection of **24-26** with TFA/CH₂Cl₂ and subsequent coupling to (*S*)-2-(morpholine-4-carboxamido)-3-phenylpropanoic acid (Mu-Phe-OH), which corresponds to the previously described P₂-P₃ position of our target compounds,^[30] yields alcohols **28-30**. Finally, oxidation of the secondary alcohol under Swern conditions provided the target compounds **1, 4** and **5**. The complete reaction scheme was repeated with hPhe (n = 2). In this case, similar to McKerrow, we introduced both the morpholine and *N*-methylpiperazine moiety in P₃-position, generating target compounds **2** and **3**. As supported by HPLC and ¹H-NMR, compounds **1-5** all revealed to have undergone a degree of racemisation due to the loss of chirality of the P₁ amino acid. (diastereomeric ratios ranging from 1:1 to 4:1) In some cases these isomers were completely or partially separated, generating two products (e.g. **1a** and **1b**). Detailed analysis of each product can be found in the experimental part.

The synthesis of target compounds **6-9** relies on a 1,3-dipolar cycloaddition with in this case the dipolarophile connected to the peptidic part of the molecule and not the 1,3-dipole as

before (Scheme 2). Treatment of Weinreb amide **37** with ethynylmagnesium bromide followed by quenching excess Grignard reagent with aqueous NaHSO₄ solution afforded the key intermediate ynone **38**.

Phenylisoxazole **6** was synthesized by cycloaddition of ynone **38** and phenylnitromethane **34**. The latter was made from the reaction of sodium nitrite with benzyl bromide.^[31] Phenylnitromethane **34** was converted into the nitrile oxide using Mukaiyama's method followed by the addition of ynone **38**. The cycloaddition product **39** was isolated in moderate yield, and directly coupled to Mu-Phe-OH using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/hydroxybenzotriazole (HOBt) as coupling reagent. In the synthesis towards phenyltriazole **7** we made use of a Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition reaction,^[32] performed between ynone **38** and phenylazide **36**. Phenyl azide is prepared (carefully) by the diazotization of phenylhydrazine with nitrous acid^[33] and used as such in the next step, no distillation or other purification is performed, which resulted in a rather moderate yield for the cycloaddition itself. After coupling, target compound **7** was isolated. In both cases (**6** and **7**) a diastereomeric mixture was obtained while the cycloaddition appeared to have taken place regioselectively. Triazoles **8** and **9** were also synthesized via a Cu-catalyzed azide-alkyne 1,3-dipolar cycloaddition reaction, performed between ynone **38** and benzyloxymethylazide but are coupled using the method described by Maryanoff,^[34] which resulted in a reduced degree of racemization. Removal of the benzyloxymethyl group, was obtained by hydrogenolysis of **8** at palladium on carbon. Subsequent elimination of formaldehyde however did not require the described basic conditions.^[35]

Target compounds **10-14** were synthesized by two similar routes, which are illustrated for the synthesis of (benzo)thiazoles **10-12** and (benz)oxazoles **13-14** in Scheme 4 and Scheme 5, respectively. In the first route the (benzo)thiazoleheterocycle was introduced by reacting the Weinreb amide **37** with an appropriate lithioheterocycle at low temperature. Excess lithium reagent was required to overcome quenching caused by the exchangeable protons in the substrate. Hence, treatment of Weinreb amide with 2-4 mol equiv of lithioheterocycle at -78°C provided the corresponding ketone.

Thiazole and benzothiazole are commercially available and yielded compounds **47** and **48** in this way. 5-Phenylthiazole **46** was synthesized in two steps following a described procedure.^[36] Treatment with an excess of lithium diisopropylamide and consecutive addition of **37** generated intermediate **49**, which was directly coupled to Mu-Phe-OH, furnishing target compound **12** as a mixture of two diastereomers (ratio 2:1). The different coupling conditions used towards the end compounds **10-11** are again a result of modifying an older less efficient direct coupling strategy to a more recently applied procedure.^[37] Remarkably, no sign of racemization was seen in target compounds **10-11**. In a second route the heterocycles, 5-phenyloxazole and benzoxazole, were introduced by reacting phenylalaninal **18** with an appropriate metalated heterocycle^[40] at low temperature (Scheme 5). Coupling and Swern oxidation afforded target compounds **13** and **14**.

Oxadiazole target compound **15** was synthesized in a stepwise linear pathway depicted in Scheme 6. Lewis acid mediated cyanohydrin formation using trimethylsilylcyanide^[41] converted phenylalaninal **18** into cyanohydrin **57** as a diastereomeric mixture. This was converted to *N*-hydroxy-amidine **58** in the presence of 50% aqueous hydroxylamine in methanol at reflux.^[42] Acylation of the *N*-hydroxy-amidine in the presence of benzoic acid and DCC (dicyclohexylcarbodiimide)-HOBt followed by thermal intramolecular cyclization afforded the 1,2,4-oxadiazole **59**. After coupling to Mu-Phe-OH, the diastereomeric alcohol **60** was oxidized using Swern conditions affording target compound **15**.

Biochemical evaluation

All synthesized compounds were tested as competitive inhibitors of *L. mexicana* cysteine protease CPB2.8 Δ CTE, a recombinant cathepsin L-like cysteine protease lacking the C-terminal extension.^[10] A competition assay was used to obtain IC₅₀ values (described in the experimental section) involving Z-FR-pNA as a colorigenic substrate. K_i values of the reversible inhibitors (**1-15**) were calculated (Table 1). Biochemical results of the presented target ketoheterocycle inhibitors **1-17** on CPB2.8 Δ CTE cover a broad activity range (IC₅₀), from a high micromolar range down to double-digit nanomolar potency (Table 1). A first interpretation of these results clearly indicates that the isoxazoles **1-3** and especially the oxadiazole **15** are promising potent inhibitors of CPB2.8 Δ CTE. The 1,2,4-oxadiazole **15** revealed an IC₅₀ of 51 nM. Also the triazole **7a** and the oxazole **13** showed activities in the nanomolar range. Furthermore, the importance of the previously discussed ring nitrogen in the heterocyclic ring is exemplified by comparing the structurally similar isoxazoles **1** and **6**. The latter is lacking this sp²-ring nitrogen and reveals a 10-fold drop in activity, indicating the importance of the hydrogen bond with the active site imidazole of histidine. Variation of the substituents in 5 position of isoxazole **1**, **4** and **5** show a 30-fold drop in activity between phenyl- and benzyl substitution, a trimethylsilyl substitution is not tolerated by the enzyme. The irreversible inhibitors **Mu-K11777** and **N-Me-K11777** are low nanomolar inhibitors of CPB2.8 Δ CTE (Table 1) but their mode of inhibition does not allow a straightforward comparison with reversible inhibitors.

A rather remarkable observation is that the two diastereomers **1a** and **1b** display similar inhibitory activities, while in other cases (**2a**, **2b** and **7a**, **7b**) the distinct diastereomers do match up with the intuitively expected stereoselectivity of the enzyme. A possible explanation to justify the observed activity of **1a** and **1b** is that stereomutation might occur during biochemical assays. Maryanoff et al. evaluated the epimerization of some ketoheterocycles in enzyme assay conditions and revealed time-dependent epimerization from the *L*- to the *D*-diastereomer. The rate of conversion is compound- and pH-dependent, and complete racemates are obtained in timeframes ranging from 1 h to 16 days.^[37]

Compound **15** was shown to inhibit papain with an IC₅₀ of 200 $\pm$ 15 nM, indicating that the compound has a broad specificity for cathepsin L family proteins. The kinetics of inhibition of CPB2.8 Δ CTE by compound **15** was investigated further. Rates determined with 20 nM CPB2.8 Δ CTE and various concentrations of inhibitor and substrate were analysed graphically using the method described by Dixon for tight-binding inhibitors.^[38] K_i^{app} values increased with increasing substrate concentration indicating that **15** is a competitive inhibitor. The K_i of CPB2.8 Δ CTE for **15**, calculated with this method, was 3.9 $\pm$ 0.6 nM (*n* = 4). A similar value for K_i was obtained when the same data were fitted to Morrison's equation^[39] and with the Cheng-Prusoff equation (K_i = 1.6 nM, Table 1). Inhibition showed slow-binding behaviour and required pre-incubation of **15** with the enzyme before addition of substrate. No inhibition was detected when reactions were started by addition of enzyme whereas the standard pre-incubation period of 10 minutes used in this study was found to be sufficient to achieve maximum inhibition (data not shown). Careful examination of the progress curves obtained after pre-incubation with **15** showed an initial lag period of about 2 minutes in which the rate increased gradually before a linear rate was established. These observations indicate that **15** is a slow, tight binding inhibitor of CPB2.8 Δ CTE.

Biological evaluation

In order to determine if inhibition of parasite cysteine proteases by these novel α -ketoheterocycle inhibitors is efficient to arrest their role in parasitic metabolism and consequently kill the parasite, the target compounds were screened in vitro on a panel of parasites consisting of *Trypanosoma brucei brucei* (bloodstream form), *Leishmania*

infantum (intracellular amastigotes), *Trypanosoma cruzi* (intracellular amastigotes) and *Plasmodium falciparum* (ring stage and schizont). *L. infantum* was chosen as the test *Leishmania* species as it is a member of the *L. donovani* complex, which causes the most severe human disease. To assess non-specific toxicity, all compounds were also evaluated for cytotoxicity on a human cell line (MRC-5). A tested compound is regarded moderately active when the IC₅₀-value is in the range of 1-16 μ M, and highly active when IC₅₀ is lower than 1 μ M. A compound is considered as non-toxic when the CC₅₀ (50% cytotoxic concentration) is higher than 32 μ M, highly toxic when CC₅₀ is lower than 4 μ M, values in between indicate moderate toxicity (Table 2). Compounds with adequate in vitro selectivity and potency can be evaluated in matching in vivo models.

Despite the nanomolar inhibition of the *L. mexicana* enzyme CPB2.8, only compound **11** showed a moderate activity towards *L. infantum* (IC₅₀=12.70 μ M) in the in vitro screening assays. Also the McKerrow compounds **Mu-K11777** and **N-Me-K11777** do not show any in vitro activity against *L. infantum*, although literature reports activity of **Mu-K11777** on amastigotes of *L. major* and *L. tropica*.^[43,44] Both vinyl sulfone inhibitors confirm their reported activity on *T. cruzi* and showed to be highly active on *T. b. brucei*.^[45] Compounds **4**, **5a**, **6**, **11** and **12** showed moderate antiparasitic activity towards *T. b. brucei*. In addition, compounds **6**, **7b**, **11**, **12** and **N-Me-K11777** also showed a moderate activity towards *P. falciparum*.

The question arises as to whether CPB is as good a target as cruzain? Obviously there are differences between the roles of the enzymes and so their value as drug targets. Literature reports clearly show that promastigotes of *L. mexicana* mutants lacking the multicopy CPB cysteine proteinase genes (Δ *cpb*) are markedly less able than wild-type parasites to infect macrophages in vitro.^[46] However, this is not the case for amastigotes, although the enzymes can be targeted by inhibitors that affect parasite survival in macrophages.^[46] Also, there are differences between *L. mexicana* (used in the enzymatic assays) and *L. infantum* (used for the biological assays), including the levels of CPB activity^[47], although the enzyme is thought to be important in both species. One explanation for the observed lack of activity of both the synthesized ketoheterocycles and the McKerrow compounds on *L. infantum* might relate to the timing of administration of the compounds, as it has been shown that the effect of CPB inhibitors on intracellular amastigotes varies though the intracellular infection.^[47] We have confirmed that both McKerrow compounds have in vitro activity against *T. cruzi* and *T. b. brucei*, whereas our compounds were inactive against these parasites. This could be because our ketoheterocycles are not active on cruzain, enter the host mammalian cells poorly, or, as once claimed by McKerrow, one needs irreversible enzyme inhibition to successfully cure the parasitic infections.^[48]

Conclusion

Inspired by the success of the vinyl sulfone cysteine protease inhibitors (CPIs) described by McKerrow, we designed and synthesized a series of reversible peptidyl α -ketoheterocycle-based CPIs. Enzymatic inhibitory assays on a recombinant *L. mexicana* cysteine protease CPB2.8 revealed promising activity. Isoxazoles **1-3** and oxadiazole **15** are potent reversible inhibitors of CPB2.8; the 1,2,4-oxadiazole **15** revealed a nanomolar potency with an K_i of 4 nM. The inhibitory trends observed in the biochemical assay are not sustained in the biological assays. In vitro screening on a panel of parasites consisting of *L. infantum*, *T. b. brucei*, *T. cruzi* and *P. falciparum* revealed moderate but selective activity for some compounds, however, not correlated with the observed enzymatic results. Further research with oxadiazole **15** as lead structure is needed to elucidate this behaviour.

Experimental Section

All starting materials were obtained from Acros Organics, Sigma Aldrich or Novabiochem. NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer (400 MHz), coupling constants are reported in Hz. Column chromatography was performed on a Flashmaster II (Jones chromatography) with Isolute columns pre-packed with silica gel. Electrospray Ionisation (ESI) mass spectra were acquired on an ion trap mass spectrometer (Bruker Daltonics Esquire™ 3000plus). LC/MS spectra were recorded on an Agilent 1100 Series LC system equipped with a C₁₈-column (2.1 × 50 mm, 5 μm, Supelco, Sigma-Aldrich) coupled to the Bruker Daltonics Esquire™ 3000plus mass spectrometer, solvent A: H₂O with 0.1% formic acid, solvent B: ACN with 0.1% formic acid; gradient system: 5% B towards 100% B in 23 min, 0.2 mL/min. HPLC was performed on a Gilson instrument equipped with a C₁₈-column (4.6 mm × 25 cm, 5 μm, Ultrasphere™ ODS), with standard UV-detection at λ = 214 nm, solvent A: H₂O with 0.1% trifluoroacetic acid, solvent B: ACN with 0.1% trifluoroacetic acid; gradient: 10% B towards 100% B in 36 min, 1 mL/min.

Biochemical assay

Enzyme source - A major cysteine protease (CPB) of *Leishmania mexicana*, that is predominantly expressed in the form of the parasite that causes disease in mammals, has been overexpressed in *Escherichia coli* and purified from inclusion bodies to apparent homogeneity. The CPB enzyme, CPB2.8, was expressed as an inactive pro-form lacking the characteristic C-terminal extension (CPB2.8ΔCTE). Purification and activation of the recombinant enzyme was described previously.^[10] The enzyme concentration determined by active site titration with E-64 was found to be 15 μM. The active enzyme represented about 30% of the total recombinant enzyme, as reported previously.^[10] Papain (10 μmol/min/mg protein) was obtained from Sigma-Aldrich

Assays - Compounds were dissolved in DMSO to a final concentration of 20 mg/mL, aliquoted and stored at -20°C. CPB2.8ΔCTE assays were performed in 1 ml of 100 mM sodium acetate (pH 5.0), 2 mM EDTA, 1 mM DTT, 40 nM enzyme, various concentrations of inhibitor, 300 μM Z-FR-pNA. The final concentration of DMSO was 1.5% (v/v). The enzyme and inhibitor were pre-incubated at 30°C for 10 minutes before starting the reaction by addition of the substrate, Z-FR-pNA*. The absorbance at 410 nm was measured continuously for 5 minutes at 30°C. Progress curves were linear for 2-3 minutes (up to 10% conversion of substrate). The percentage inhibition with 4 different concentrations of each compound (20, 2, 0.2 and 0.02 μg/ml) were determined in a preliminary experiment. IC₅₀ values were then obtained for selected compounds with 7 different concentrations of the inhibitor and a control reaction containing DMSO only. K_i values were calculated with the Cheng-Prusoff equation ($K_i = IC_{50}/(1 + [S]/K_m)$) with S = 300 μM and K_m = 10 μM. The equation applies to reversible competitive inhibitors and cannot be used to calculate K_i values for the irreversible inhibitors. The K_i of CPB2.8ΔCTE for compound **15** was determined by measuring the rates of reactions containing 20 nM enzyme with 4 different concentrations of substrate (10 – 100 μM) and 10 different concentrations of inhibitor (0 – 200 nM). Two different methods were used to determine the K_i. (1) Values for K_i^{app} at each substrate concentration were determined from plots of the reaction velocity against inhibitor concentration using the method described by Dixon for tight-binding inhibitors.^[38] The K_i was then calculated using the equation $K_i = K_i^{app}/(1 - [S]/K_m)$. (2) The data was also analysed by an independent method in which the K_i was derived by fitting the reaction rates to Morrison's equation^[39] with GraphPad Prism 5.0. The K_m of CPB2.8ΔCTE for Z-FR-pNA, determined with 20 nM CPB2.8ΔCTE and variable concentration of Z-FR-pNA (2 – 200 μM), was 10 ± 1 nM. The time dependence of inhibition was investigated by incubating CPB2.8ΔCTE (20 nM) for various times with 50 nM compound **15** before starting the

reaction by addition of Z-FR-pNA to a final concentration of 300 μM . Papain assays were performed in 1 ml of 50 mM Na_2HPO_4 (pH 6.5), 2 mM EDTA, 5 mM L-cysteine, 0.5 mg/ml papain, various concentrations of inhibitor and 100 μM Z-FR-pNA. The enzyme was pre-incubated with inhibitor for 10 minutes at 30°C before starting the reaction by addition of substrate. The absorbance at 410 nm was measured continuously for 10 mins at 30°C and rates were calculated from the progress curves. The rate obtained without inhibitor was linear for 2-3 min and similar to that obtained with 20 nM CPB2.8 Δ CTE. The IC_{50} value of compound **15** with papain was obtained with 7 different concentrations of the inhibitor (20 nM – 20 μM) and a control reaction containing DMSO only.

*Z-Phe-Arg-pNA.HCl (Z = Cbz = Benzyloxycarbonyl; pNA = p-nitroanilide), chromogenic substrate for cathepsins B, K, L, and S, papain, and trypsin.

Biological assay

The strains used for antiparasitic in vitro screening included *T. b. brucei* Squib 427 (bloodstream form), *T. cruzi* Tulahuen CL2 β -galactosidase (intracellular amastigotes), *L. infantum* MHOM/MA(BE)/67 (intracellular amastigotes) and *P. falciparum* GHA (schizont and ring stage). MRC-5_{SV2} cells (human fibroblasts) were used for evaluation of cytotoxicity. All cultures and assays were conducted at 37°C.

Assays - Compound stock solutions were prepared in 100% DMSO at 20mM and kept at room temperature. The compounds are serially pre-diluted (2-fold or 4-fold) in DMSO followed by further dilution in demineralized water to assure a final 'in-test' DMSO concentration of <1%. Assays are performed in sterile 96-well microtiter plates, each well containing 10 μL of the compound diluted together with 190 μL of the parasite or cell suspension. Parasite growth is compared to untreated-infected controls (100% growth) and medium-control wells (0% growth). The compounds are tested at five concentrations (64, 16, 4, 1 and 0.25 μM). After an incubation period, parasite multiplication is assessed by different methods, for example by measuring colour change or fluorescence after the addition of a dye or fluorescent marker.^[49]

Chemistry

General Procedures

Procedure A (Boc deprotection and coupling reaction): The Boc-protected compound was dissolved in CH_2Cl_2 (2 mL). Upon addition of trifluoroacetic acid (2 mL) the solution turned darker brown. The mixture was left stirring at room temperature for 2 h, and then the residue was dried under reduced pressure. The deprotected compound was detected using mass spectroscopy. The TFA-salt was dissolved in DMF and stirred at room temperature. The free acid (1 eq), TBTU (1.1 eq) and NEt_3 (2.2 eq) were added to the solution. After stirring overnight, a fivefold volume of water was added and the mixture was extracted with ethyl acetate (3×20 mL). The combined organic layers were washed with 2N HCl (2×50 mL), water (1×50 mL), saturated NaHCO_3 (2×50 mL), and brine (2×50 mL). After drying over Na_2SO_4 , the solution was filtered and concentrated by rotary evaporation. This crude mixture was then subjected to flash chromatography.

Procedure B (Swern oxidation): Dimethyl sulfoxide (4 eq) was added to a solution of oxalyl chloride (2 eq) in dry dichloromethane, which was precooled to -78°C and kept under nitrogen atmosphere. The mixture was stirred at -78°C for 10 min. The secondary alcohol (1 eq) dissolved in dry dichloromethane was added dropwise to the solution via cannula and the mixture was stirred at -78°C for 30 min. Triethylamine (4 eq) was added, and the mixture was allowed to warm up to 0°C and stirred for 2 h. Afterwards the solution

was diluted with ethyl acetate, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. This crude mixture was then subjected to flash chromatography.

Procedure C (Cycloaddition using nitrile oxide): Phenylisocyanate (2 eq) and dry triethylamine (few drops) were added to a solution of nitroalcohol (1 eq) and the appropriate acetylene (1 eq) in dry toluene (5 mL). The solution was heated at 90°C for 18 h, after cooling the reaction was quenched by adding few drops of water, and stirring was continued for 1 h. The reaction mixture was filtered, dried over anhydrous Na_2SO_4 and toluene was removed under reduced pressure. To avoid hazards during purification the trimethylsilyl protecting group was removed by acetic acid/water (80:20) or 10% citric acid in methanol. This crude mixture was then subjected to flash chromatography (hexane/ethyl acetate 1:1 towards 100% ethyl acetate in 35 min).

(S)-tert-Butyl 1-oxo-3-phenylpropan-2-ylcarbamate (18): (Step a, synthesis of Weinreb amide **37**) Boc-protected phenylalanine (8 g, 30.18 mmol), *N,O*-dimethylhydroxylamine (2.94 g, 30.18 mmol) and TBTU (10.6 g, 33.2 mmol) were dissolved in DMF (100 mL). Triethylamine (12.7 mL, 90.54 mmol) was added and the turbid solution was left stirring at room temperature. After 24 h a fivefold volume of water (500 mL) was added and the mixture was extracted with ethyl acetate (3×100 mL). The combined organic layers were then washed consecutively with 2N HCl (2×90 mL), sat. NaHCO_3 (2×90 mL) and brine (90 mL). After drying over Na_2SO_4 the solvent was removed under reduced pressure, and the product was subjected to flash column chromatography (100% hexane towards 100% ethyl acetate in 40 min). Colourless oil was obtained afterwards (9.12 g, 98%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.37 (s, 9H, Boc), 2.86 (m, 1H, CH_2Ph), 3.03 (dd, $J_F=6$ Hz, $J_2=13.6$ Hz, 1H, CH_2Ph), 3.14 (s, 3H, NCH_3), 3.64 (s, 3H, OCH_3), 4.94 (s br, 1H, CH), 5.18 (s br, 1H, NH), 7.15-7.28 (m, 5H, Ph); MS (ESI) m/z : 331 [$M+\text{Na}$] $^+$ and 639 [$2M+\text{Na}$] $^+$.

(Step b, reduction of **37** to the aldehyde) The *N*-methoxy-*N*-methylethylamide derivative (9.12 g, 29.61 mmol) was dissolved in dry THF (60 mL) and cooled to -10°C using an ice/salt-bath. Under vigorous stirring, LiAlH_4 (1.12 g, 29.61 mmol) was added in 6 portions over a period of 40 min. The internal reaction temperature may not exceed -5°C during the addition. After addition the reaction mixture was left stirring at this temperature for another 2 h. The mixture was carefully quenched by dropwise addition of a 1M KHSO_4 solution. After the addition of diethyl ether (300 mL), the aqueous layer was separated and extracted with diethyl ether (3×200 mL). The combined organic layers were successively washed with 1N HCl (1×80 mL), water (1×80 mL) and brine (1×80 mL). After drying over Na_2SO_4 the solvent was removed under reduced pressure, and the product was used in the next step without further purification. A white solid was obtained. (6.87 g, 93%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.32 (s, 9H, Boc), 2.99 (m, 2H, CH_2Ph), 4.25 (s br, 1H, CH), 5.17 (s br, 1H, NH), 7.05-7.20 (m, 5H, Ph), 9.47 (s, 1H, CHO); MS (ESI) m/z : 304 [$M+\text{MeOH}+\text{Na}$] $^+$ observed as hemiacetal.

(S)-tert-Butyl 1-oxo-4-phenylbutan-2-ylcarbamate (19): (Step a, synthesis of Weinreb amide) Boc-protected homophenylalanine (2.9 g, 10.4 mmol), *N,O*-dimethylhydroxylamine (1.01 g, 10.4 mmol) and TBTU (3.66 g, 11.4 mmol) were dissolved in DMF (80 mL). Triethylamine (3.2 mL, 22.8 mmol) was added and the procedure was followed as described for **18**. A colourless oil was obtained (3.3 g, 99%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.46 (s, 9H, Boc), 1.83 (m, 1H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.01 (m, 1H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.64-2.80 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 3.16 (s, 3H, NCH_3), 3.62 (s, 3H, OCH_3), 4.69 (s br, 1H, CH), 5.23 (d, $J=8$ Hz, 1H, NH), 7.16-7.30 (m, 5H, Ph); MS (ESI) m/z : 345 [$M+\text{Na}$] $^+$.

(Step b, reduction to aldehyde) The *N*-methoxy-*N*-methylethylamide derivative (3.31 g, 10.28 mmol) was dissolved in dry THF (60 mL) and cooled to -10°C using an ice/salt-bath. Under

vigorous stirring, LiAlH₄ (0.52 g, 13.7 mmol) was added and the procedure was followed as described for **18**. A pale yellow oil was obtained (2.62 g, 97%); ¹H-NMR (400 MHz, CDCl₃): δ=1.38 (s, 9H, Boc), 1.76 (m, 2H, CH₂CH₂Ph), 2.62 (m, 2H, CH₂CH₂Ph), 4.14 (s br, 1H, CH), 5.13 (s br, 1H, NH), 7.08-7.22 (m, 5H, Ph), 9.45 (s, 1H, CHO); MS (ESI) *m/z*: 318 [*M*+ MeOH+Na]⁺ observed as hemiacetal.

tert-Butyl (2S)-3-hydroxy-4-nitro-1-phenylbutan-2-ylcarbamate (20): To a solution of tetra-*n*-butylammonium fluoride (1M in THF, 34.3 mL, 34.3 mmol) in dry THF (20 mL) at 0°C, a solution of nitromethane (2.22 mL, 41.2 mmol) in dry THF (20 mL) was added dropwise. After stirring the resulting solution for 5 min at 0°C a solution of Boc-Phe-H (**18**) (4.27 g, 17.15 mmol) in THF was added dropwise over 5 min and stirring was continued for 3 h. The reaction was diluted with sat. NaHCO₃ (60 mL) and extracted with diethyl ether (3 × 60 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude oil was loaded onto a silica gel column and eluted with hexane/ethyl acetate 1:2 to furnish β-nitroalcohol **3** as a yellow oil (2.88 g, 54%); ¹H-NMR (400 MHz, CDCl₃): δ=1.40 (s, 9H, Boc), 2.93 (d, *J*=7.6 Hz, 2H, CH₂Ph), 3.84 (m, 1H, CH), 4.03 (s br, 0.7H, OH), 4.32 (m, 1H, CHOH), 4.42 (s br, 2H, CH₂NO₂), 5.05 (d, *J*=9.2 Hz, 1H, NH), 7.20-7.32 (m, 5H, Ph); MS (ESI) *m/z*: 333 [*M*+Na]⁺.

tert-Butyl (3S)-2-hydroxy-1-nitro-5-phenylpentan-3-ylcarbamate (21): Tetra-*n*-butylammonium fluoride (1M in THF, 20.5 mL, 20.5 mmol) in dry THF (20 mL), nitromethane (1.32 mL, 24.6 mmol) and Boc-hPhe-H **19** (2.7 g, 10.3 mmol) were reacted following the procedure as described for **20**, a yellow oil was obtained (2.88 g, 54%); ¹H-NMR (400 MHz, CDCl₃): δ=1.45 (s, 9H, Boc), 1.90 (m, 2H, CH₂CH₂Ph), 2.70 (m, 2H, CH₂CH₂Ph), 3.63 (m, 1H, CH), 4.36 (m, 1H, CHOH), 4.44 (m, 2H, CH₂NO₂), 4.90 (d, *J*=8.8 Hz, 0.7H, NH), 7.16-7.31 (m, 5H, Ph); MS (ESI) *m/z*: 347 [*M*+Na]⁺.

tert-Butyl (2S)-4-nitro-1-phenyl-3-(trimethylsilyloxy)butan-2-ylcarbamate (22): Trimethylsilyl chloride (2.1 mL, 16.6 mmol) and triethylamine (2.33 mL, 16.6 mmol) were combined with 10 mL of dry THF, the mixture turned milky white. The solution was cooled to 0°C using an ice bath. A solution of the nitroalcohol **20** (2.57 g, 8.3 mmol) in dry THF (20 mL) was added dropwise over a period of 10 min using an additional funnel. The mixture was stirred in an ice bath for 24 h, during which temperature slowly raised to room temperature. The reaction was quenched with 15 mL of ice-water, and the mixture was extracted with diethyl ether (3 × 30 mL). The combined organic extracts were dried over anhydrous Na₂SO₄ and evaporated under reduced pressure to yield a colourless oil. (2.76 g, 87%); ¹H-NMR (400 MHz, CDCl₃): δ=0.16 (s, 9H, OTMS), 1.35 (s, 9H, Boc), 2.81 (m, 2H, CH₂Ph), 3.68 (m, 1H, CH), 4.37 (m, 2H, CH₂NO₂), 4.47 (m, 1H, HCOTMS), 5.24 (d, *J*=9.2 Hz, 0.6H, NH), 7.10-7.25 (m, 5H, Ph); MS (ESI) *m/z*: 405 [*M*+Na]⁺.

tert-Butyl (3S)-1-nitro-5-phenyl-2-(trimethylsilyloxy)pentan-3-ylcarbamate (23): Trimethylsilyl chloride (1.7 mL, 13.3 mmol), triethylamine (1.87 mL, 13.3 mmol) were combined with a solution of nitroalcohol **21** (2.16 g, 6.66 mmol) as described for compound **22** to yield a colourless oil. (2.64 g, 88%); ¹H-NMR (400 MHz, CDCl₃): δ=0.1 (s, 9H, OTMS), 1.48 (s, 9H, Boc), 1.85 (m, 2H, CH₂CH₂Ph), 2.59-2.74 (m, 2H, CH₂CH₂Ph), 3.72 (m, 1H, CH), 4.35 (m, 2H, CH₂NO₂), 4.42 (m, 1H, HCOTMS), 4.84 (d, *J*=9.6 Hz, 0.6H, NH), 7.16-7.30 (m, 5H, Ph); MS (ESI) *m/z*: 419 [*M*+Na]⁺.

tert-Butyl (2S)-1-hydroxy-3-phenyl-1-(5-phenylisoxazol-3-yl)propan-2-ylcarbamate (24): Compound **22** (0.2 g, 0.52 mmol) and phenylacetylene (0.06 mL, 0.52 mmol) were used in procedure C to obtain compound **24** in moderate yield. (90 mg, 43%); ¹H-NMR (400 MHz, CDCl₃): δ=1.38 (s, 9H, Boc), 3.03 (m, 2H, CH₂Ph), 4.02 (s br, 0.7H, OH), 4.12 (m, 1H,

CH), 4.91 (d, $J=3.6$ Hz, 1H, CHOH), 5.05 (d, $J=9.2$ Hz, 1H, NH), 6.57 (s, 1H, isoxazole-H), 7.11-7.75 (m, 10H, $2 \times \text{Ph}$); MS (ESI) m/z : 395 $[M+H]^+$.

tert-Butyl (2S)-1-(5-benzylisoxazol-3-yl)-1-hydroxy-4-phenylbutan-2-ylcarbamate (25):

Compound **22** (1.06 g, 2.67 mmol) and 3-phenyl-1-propyn (0.4 mL, 3.3 mmol) were used in procedure C to obtain compound **25** as a yellowish oil. (0.32 g, 28%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.38$ (s, 9H, Boc), 1.92 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.71 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 3.65 (s br, 1H, OH), 3.82 (m, 1H, CH), 4.04 (s, 2H, PhCH_2 -isoxazole), 4.83 (s br, 1H, CHOH), 4.92 (d, $J=9.2$ Hz, 1H, NH), 5.95 (s, 1H, isoxazole-H), 7.04-7.46 (m, 10H, $2 \times \text{Ph}$); MS (ESI) m/z : 445 $[M+H]^+$.

tert-Butyl (2S)-1-hydroxy-3-phenyl-1-[5-(trimethylsilyl)isoxazol-3-yl]propan-2-ylcarbamate (26):

Compound **22** (0.5 g, 1.3 mmol) and trimethylsilylacetylene (0.18 mL, 1.3 mmol) were used in procedure C to obtain compound **26** in moderate yield. (260 mg, 51%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=0.32$ (s, 9H, TMS), 1.34 (s, 9H, Boc), 2.91-3.04 (m, 2H, CH_2Ph), 4.08 (m, 1H, CH), 4.91 (d, $J=3.6$ Hz, 1H, CHOH), 5.01 (d, $J=9.2$ Hz, 1H, NH), 6.46 (s, 1H, isoxazole-H), 7.19-7.39 (m, 10H, $2 \times \text{Ph}$); MS (ESI) m/z : 391 $[M+H]^+$.

tert-Butyl (2S)-1-hydroxy-4-phenyl-1-(5-phenylisoxazol-3-yl)butan-2-ylcarbamate (27):

Compound **23** (1.5 g, 3.8 mmol) and phenylacetylene (0.41 mL, 3.8 mmol) were used in procedure C to obtain compound **27** in moderate yield (0.55 g, 37%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.40$ (s, 9H, Boc), 1.97 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.76 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 3.63 (s br, 1H, OH), 3.95 (m, 1H, CH), 4.95 (s br, 1H, CHOH), 5.17 (d, $J=9.6$ Hz, 1H, NH), 6.58 (s, 1H, isoxazole-H), 7.15-7.73 (m, 10H, $2 \times \text{Ph}$); MS (ESI) m/z : 431 $[M+H]^+$.

N-[(2S)-1-[(2S)-1-Hydroxy-3-phenyl-1-(5-phenylisoxazol-3-yl)propan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (28):

Compound **24** (80 mg, 0.2 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **28**. (80 mg, 69%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=2.91$ (m, 2H, CH_2Ph), 3.12 (m, 2H, CH_2Ph), 3.19 (m, 4H, CH_2N), 3.56 (m, 4H, CH_2O), 4.42 (m, 1H, CH), 4.54 (m, 1H, CH), 4.92 (d, $J=3.6$ Hz, 1H, CHOH), 4.98 (d, $J=9.2$ Hz, 1H, NH), 6.60 (s, 1H, isoxazole-H), 7.02-7.76 (m, 15H, $3 \times \text{Ph}$); MS (ESI) m/z : 577 $[M+Na]^+$.

N-[(2S)-1-[(2S)-1-(5-Benzylisoxazol-3-yl)-1-hydroxy-4-phenylbutan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (29):

Compound **25** (0.32 g, 0.76 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **29**. (0.27 g, 61%); HPLC ($\lambda = 214$ nm) t_R : 24.11 min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.86$ (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.57 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.88-3.03 (m, 4H, CH_2Ph), 3.21 (m, 4H, CH_2N), 3.51 (m, 4H, CH_2O), 3.98 (s, 2H, PhCH_2 -isoxazole), 4.16 (m, 1H, CH), 4.69 (m, 1H, CH), 4.82 (s br, 1H, CHOH), 5.22 (d, $J=7.2$ Hz, 1H, NH), 5.94 (s, 1H, isoxazole-H), 6.93-7.31 (m, 15H, $3 \times \text{Ph}$); MS (ESI) m/z : 605 $[M+Na]^+$.

N-[(2S)-1-[(2S)-1-Hydroxy-3-phenyl-1-[5-(trimethylsilyl)isoxazol-3-yl]propan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (30):

Compound **26** (0.26 g, 0.67 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **30**. (0.21 g, 57%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=0.29$ (s, 9H, TMS), 2.79-3.08 (m, 4H, $2 \times \text{CH}_2\text{Ph}$), 3.19 (m, 4H, CH_2N), 3.54 (m, 4H, CH_2O), 4.46 (m, 1H, CH), 4.55 (m, 1H, CH), 4.97 (s br, 1H, CHOH), 5.31 (s br, 1H, NH), 6.53 (s, 1H, isoxazole-H), 7.05-7.39 (m, 10H, $2 \times \text{Ph}$); MS (ESI) m/z : 573 $[M+Na]^+$.

N-[(2S)-1-[(2S)-1-Hydroxy-4-phenyl-1-(5-phenylisoxazol-3-yl)butan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (31):

Compound **27** (0.25 g, 0.61 mmol)

was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **31**. (0.18 g, 54%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.82 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.58 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.87-3.03 (m, 4H, CH_2Ph), 3.18 (m, 4H, CH_2N), 3.52 (m, 4H, CH_2O), 3.79 (s br, 1H, OH), 4.11 (m, 1H, CH), 4.84 (m, 1H, CH), 4.92 (d, J =6.8 Hz, 1H, CHOH), 6.47 (s, 1H, isoxazole-H), 6.83 (d, J =9.2 Hz, 1H, NH), 7.02-7.76 (m, 15H, $3 \times \text{Ph}$); MS (ESI) m/z : 591 $[\text{M}+\text{Na}]^+$.

***N*-{[(2*S*)-1-[(2*S*)-1-Hydroxy-4-phenyl-1-(5-phenylisoxazol-3-yl)butan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]-4-methylpiperazine-1-carboxamide (32)**: Compound **27** (0.25 g, 0.61 mmol) was used in procedure A, coupled to *N*-Me-Phe-OH and yielded coupled product **32** (40 mg, 46%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.88 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.14 (s, 3H, NCH_3), 2.18 (m, 4H, H_3CNCH_2), 2.56 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.85-3.01 (m, 4H, CH_2Ph), 3.20 (m, 4H, CH_2N), 4.11 (m, 1H, CH), 4.50 (q, J =6.8 Hz, 1H, CH), 4.84 (d, J =4.8 Hz, 1H, CHOH), 4.98 (d, J =7.2 Hz, 1H, NH), 6.50 (s, 1H, isoxazole-H), 6.99-7.19 (m, 10H, $2 \times \text{Ph}$), 7.35 (m, 3H, Ph), 7.65 (m, 2H, Ph); MS (ESI) m/z : 582 $[\text{M}+\text{H}]^+$.

***N*-{[(*S*)-1-Oxo-1-[(*S*)-1-oxo-3-phenyl-1-(5-phenylisoxazol-3-yl)propan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (1)**: Compound **28** (80 mg, 0.14 mmol) was used in procedure B and yielded keto-isoxazole **1**. (40 mg, 50%) Both $^1\text{H-NMR}$ as HPLC analysis showed the presence of two isomers **1a** and **1b**, which were separated using preparative thin layer chromatography. **Isomer 1a**: Off-white solid; HPLC (λ = 214 nm): t_R =27.04 min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.81-2.99 (m, 2H, CH_2Ph), 3.01-3.30 (m, 2H, CH_2Ph), 3.20 (m, 4H, CH_2N), 3.56 (m, 4H, CH_2O), 4.51 (q, J =6 Hz, 1H, CH), 4.95 (d, J =6.8 Hz, 1H, NH), 5.53 (m, 1H, CH), 6.43 (d, J =6.8 Hz, 1H, NH), 6.83 (s, 1H, isoxazole-H), 6.86 (m, 2H, Ph), 7.10-7.23 (m, 8H, Ph), 7.45 (m, 3H, Ph), 7.76 (m, 2H, Ph); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ = 37.84 (CH_2Ph), 38.49 (CH_2Ph), 43.98 (CH_2NCH_2), 55.39 (chiral CH), 57.63 (chiral CH), 66.37 (CH_2OCH_2), 98.33 (isoxazole C_4), 126.01, 126.51, 127.01, 127.07, 128.53, 128.67, 129.22, 129.34, 129.49, 130.95, 135.47, 136.83 ($3 \times \text{Ph}$), 156.88 (isoxazole C_3), 160.48 (C=O, urea), 171.29 (isoxazole C_5), 171.76 (C=O amide), 191.14 (C=O, alpha to het.) ppm; MS (ESI) m/z : 575 $[\text{M}+\text{Na}]^+$. **Isomer 1b**: White solid; HPLC (λ = 214 nm): t_R =26.66 min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.96 (d, J =6.8 Hz, 2H, CH_2Ph), 3.07-3.17 (m, 2H, CH_2Ph), 3.20 (m, 4H, CH_2N), 3.55 (m, 4H, CH_2O), 4.56 (q, J =6.8 Hz, 1H, CH), 4.93 (d, J =7.6 Hz, 1H, NH), 5.62 (m, 1H, CH), 6.70 (d, J =7.6 Hz, 1H, NH), 6.79 (s, 1H, isoxazole-H), 6.87 (m, 2H, Ph), 7.04-7.23 (m, 8H, Ph), 7.44 (m, 3H, Ph), 7.74 (m, 2H, Ph); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ =37.88 (CH_2Ph), 38.37 (CH_2Ph), 43.98 (CH_2NCH_2), 55.36 (chiral CH), 57.48 (chiral CH), 66.36 (CH_2OCH_2), 98.33 (isoxazole C_4), 125.98, 126.49, 126.96, 127.17, 128.52, 128.62, 129.19, 129.35, 129.49, 130.92, 135.41, 136.78 ($3 \times \text{Ph}$), 156.95 (isoxazole C_3), 160.52 (C=O, urea), 171.56 (isoxazole C_5), 171.70 (C=O amide), 191.63 (C=O, alpha to het.) ppm; MS (ESI) m/z : 575 $[\text{M}+\text{Na}]^+$.

***N*-{[(*S*)-1-Oxo-1-[(*S*)-1-oxo-4-phenyl-1-(5-phenylisoxazol-3-yl)butan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (2)**: Compound **31** (0.19 g, 0.34 mmol) was used in procedure B and yielded keto-isoxazole **2**. (60 mg, 32%) Both $^1\text{H-NMR}$ and HPLC analysis showed the presence of two isomers **2a** and **2b**, which were separated using preparative thin layer chromatography. **Isomer 2a**: White solid; HPLC (λ = 214 nm): t_R =27.32 min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.05 (m, 1H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.35 (m, 1H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.61 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$), 3.00-3.16 (m, 2H, CH_2Ph), 3.31 (m, 4H, CH_2N), 3.64 (m, 4H, CH_2O), 4.62 (q, J =6.8 Hz, 1H, CH), 5.13 (d, J =7.2 Hz, 1H, NH), 5.42 (m, 1H, CH), 6.75 (d, J =7.2 Hz, 1H, NH), 6.84 (s, 1H, isoxazole-H), 7.08-7.27 (m, 10H, Ph), 7.51 (m, 3H, Ph), 7.80 (m, 2H, Ph); MS (ESI) m/z : 589 $[\text{M}+\text{Na}]^+$; LC-MS: t_R =19.1 min, m/z : 567 $[\text{M}+\text{H}]^+$. **Isomer 2b**: White solid; HPLC (λ = 214 nm): t_R = 27.52 min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =19.8 (m, 1H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.28 (m, 1H, $\text{CH}_2\text{CH}_2\text{Ph}$), 2.53 (m, 2H, $\text{CH}_2\text{CH}_2\text{Ph}$),

3.03-3.18 (m, 2H, CH₂Ph), 3.33 (m, 4H, CH₂N), 3.63 (m, 4H, CH₂O), 4.71 (q, *J*=6.8 Hz, 1H, CH), 5.21 (d, *J*=7.6 Hz, 1H, NH), 5.44 (m, 1H, CH), 6.79 (s, 1H, isoxazole-H), 6.95 (d, *J*=7.6 Hz, 1H, NH), 7.03-7.31 (m, 10H, Ph), 7.49 (m, 3H, Ph), 7.77 (m, 2H, Ph); MS (ESI) *m/z*: 589 [*M*+Na]⁺; LC-MS: *t_R*=19.1 min, *m/z*: 567 [*M*+H]⁺.

4-Methyl *N*-{(S)-1-oxo-1-[(S)-1-oxo-4-phenyl-1-(5-phenylisoxazol-3-yl)butan-2-ylamino]-3-phenylpropan-2-yl}piperazine-1-carboxamide (3): Compound **32** (60 mg, 0.1 mmol) was used in procedure B and yielded keto-isoxazole **3**. (29 mg, 48%). ¹H-NMR as HPLC analysis showed the presence of two isomers, no further efforts were done to separate these isomers. Off-white solid; HPLC (*λ*= 214 nm): *t_{R1}*=22.39 min, *t_{R2}*=22.85 min; ¹H-NMR (400 MHz, CDCl₃): δ=1.97 (m, 4H, CH₂CH₂Ph), 2.21 (s, 3H, NCH₃), 2.22 (s, 3H, NCH₃), 2.29 (m, 8H, H₃CNCH₂), 2.42-2.58 (m, 4H, CH₂CH₂Ph), 2.96-3.10 (m, 4H, CH₂Ph), 3.29 (m, 8H, CH₂N), 4.54 (q, *J*=7.2 Hz, 1H, CH), 4.60 (q, *J*=7.2 Hz, 1H, CH), 5.01 (m, 2H, 2 × NH), 5.34 (m, 2H, 2 × CH), 6.72 (s, 1H, isoxazole-H), 6.76 (s, 1H, isoxazole-H), 7.00-7.23 (m, 20H, Ph), 7.42 (m, 6H, Ph), 7.73 (m, 4H, Ph); MS (ESI) *m/z*: 602 [*M*+Na]⁺; LC-MS *t_{R1}*=16.3 min, *m/z*: 580 [*M*+H]⁺, *t_{R2}*=16.8 min, *m/z*: 580 [*M*+H]⁺.

***N*-{(S)-1-[(d)-1-(5-Benzylisoxazol-3-yl)-1-oxo-4-phenylbutan-2-ylamino]-1-oxo-3-phenylpropan-2-yl}morpholine-4-carboxamide (4):** Compound **29** (0.27 g, 0.46 mmol) was used in procedure B and yielded keto-isoxazole **4**. Afterwards ¹H-NMR analysis showed the presence of two isomers (ratio 2:1), only the major isomer could be isolated with an acceptable purity level (>95%), and yielded keto-isoxazole **4** (60 mg, 32%); HPLC (*λ*=214 nm) *t_R*=27.09 min; ¹H-NMR (400 MHz, CDCl₃): δ=2.01 (m, 1H, CH₂CH₂Ph), 2.31 (m, 1H, CH₂CH₂Ph), 2.58 (m, 2H, CH₂CH₂Ph), 2.99-3.14 (m, 2H, CH₂Ph), 3.31 (m, 4H, CH₂N), 3.63 (m, 4H, CH₂O), 4.13 (s, 2H, PhCH₂-isoxazole), 4.59 (m, 1H, CH), 5.07 (s br, 1H, NH), 5.36 (m, 1H, CH), 6.27 (s, 1H, isoxazole-H), 6.64 (s br, 1H, NH), 7.06-7.38 (m, 15H, Ph); MS (ESI) *m/z*: 603 [*M*+Na]⁺; LC-MS: *t_R*=19.0 min, *m/z*: 581 [*M*+H]⁺.

***N*-{(S)-1-Oxo-1-[(S)-1-oxo-3-phenyl-1-[5-(trimethylsilyl)isoxazol-3-yl]propan-2-ylamino]-3-phenylpropan-2-yl}morpholine-4-carboxamide (5):** Compound **30** (0.19 g, 0.35 mmol) was used in procedure B and yielded keto-isoxazole **5**. (40 mg, 50%) Both ¹H-NMR as HPLC analysis showed the presence of two isomers, which were separated only partly (**5a** is 100% pure, **5b** is a 1:1 mix) using preparative thin layer chromatography. **Product 5a**: Light yellow solid; ¹H-NMR (400 MHz, CDCl₃): δ=0.39 (s, 9H, TMS), 2.97 (m, 2H, CH₂Ph), 3.06-3.32 (m, 2H, CH₂Ph), 3.26 (m, 4H, CH₂N), 3.61 (m, 4H, CH₂O), 4.57 (m, 1H, CH), 5.07 (d, 1H, *J*= 6.8 Hz, NH), 5.60 (m, 1H, CH), 6.61 (d, *J*=6.4 Hz, 1H, NH), 6.83 (s, 1H, isoxazole-H), 6.93 (m, 2H, Ph), 7.16-7.27 (m, 8H, Ph); MS (ESI) *m/z*: 571 [*M*+Na]⁺. **Product(s) 5b**: Light yellow solid; ¹H-NMR (400 MHz, CDCl₃): δ=0.37 (s, 9H, TMS), 0.39 (s, 9H, TMS), 2.97 (m, 4H, 2 × CH₂Ph), 3.06-3.32 (m, 4H, CH₂Ph), 3.26 (m, 8H, CH₂N), 3.61 (m, 8H, CH₂O), 4.57 (m, 1H, CH), 4.61 (m, 1H, CH), 5.02 (m, 2H, 2 × NH), 5.59 (m, 1H, CH), 5.66 (m, 1H, CH), 6.53 (d, *J*=6.4 Hz, 1H, NH), 6.77 (d, *J*=6.4 Hz, 1H, NH), 6.79 (s, 1H, isoxazole-H), 6.83 (s, 1H, isoxazole-H), 6.92 (m, 4H, Ph), 7.08-7.26 (m, 16H, Ph); MS (ESI) *m/z*: 571 [*M*+Na]⁺.

(Nitromethyl)benzene (34): Benzyl bromide (3.6 mL, 30 mmol) was poured into a stirred mixture of DMF (60 mL), sodium nitrite (3.6 g, 52 mmol) and urea (4 g, 66 mmol) while temperature was maintained at -20 to -30°C. After five hours the reaction mixture was worked up; poured into ice-water and extracted with diethyl ether (75 mL). The aqueous layer was extracted four more times with portions of diethyl ether (40 mL) after which the extracts were washed with portions of water (50 mL) and dried over anhydrous Na₂SO₄. The solvent was removed under reduced pressure and the crude mixture was loaded onto a

silica gel column (hexane/ethyl acetate 3:1) yielding product **34** as a syrup. (1.75 g, 43%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =5.43 (s, 2H, CH_2), 7.43-7.63 (m, 5H, Ph).

Azidobenzene (36): In a 250 mL three-necked flask fitted with a stirrer, a thermometer and a dropping funnel are placed 30 mL of water and concentrated hydrochloric acid (5.5 mL, 181 mmol). The flask is surrounded by an ice-salt bath, the stirrer is started, and phenylhydrazine (0.99 mL, 10 mmol) is added dropwise. Phenylhydrazine hydrochloride separates as fine white plates. Stirring is continued, and, after the temperature has fallen to 0°C , 10 mL of ether is added, after which a previously prepared solution of sodium nitrite (1.17 g, 17.0 mmol) in 5 mL of water is added from the dropping funnel at such a rate that the temperature never rises above 5°C . This requires 20 min. The organic phase (diethyl ether) was separated, the aqueous layer was extracted with diethyl ether once more and the ether layer was kept as such. No evaporation was done to avoid explosions, TLC monitoring (hexane/ethyl acetate 3:1) versus phenylhydrazine was done and showed no starting product present after 1 h of stirring. The solution was used as such in following experiment.

(S)-tert-Butyl 3-oxo-1-phenylpent-4-yn-2-ylcarbamate (38): At -78°C 0.5M ethynylmagnesium bromide in THF (130.4 mL, 65.2 mmol) was slowly added to the stirred solution of Weinreb amide **37** (see procedure product **18**) (5.02 g, 16.3 mmol) in anhydrous THF (40 mL) under nitrogen atmosphere, and the resulting mixture was stirred at -78°C for 1 h and at room temperature for 12 h. The mixture was poured into a cold (0°C) 1M aqueous NaHSO_4 solution (150 mL) and stirred for 1 h. THF was evaporated and the aqueous residue was extracted with diethyl ether twice (2×60 mL). The combined organic layers were washed with 1M NaHSO_4 (2×50 mL), saturated aqueous NaHCO_3 (2×50 mL) and brine (2×50 mL). Afterwards the organic phase is dried over anhydrous Na_2SO_4 , the solvent was removed under reduced pressure and the crude residue is purified by flash column chromatography (hexane/ethyl acetate 2:1). (3.25 g, 73%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.42 (s, 9H, Boc), 3.16-3.29 (m, 2H, CH_2Ph), 3.43 (s, 1H, HCC), 4.68 (m, 1H, CH), 4.99 (s br, 1H, NH), 7.15-7.31 (m, 5H, Ph); MS (ESI) m/z : 296 [$M+\text{Na}$] $^+$.

(S)-tert-Butyl 1-oxo-3-phenyl-1-(3-phenylisoxazol-5-yl)propan-2-ylcarbamate (39): Phenyl isocyanate (0.48 mL, 4.4 mmol) and dry triethylamine (few drops) were added to a solution of (nitromethyl)benzene (**34**) (0.45 g, 3.3 mmol) in dry toluene (10 mL). The solution was heated at 50°C , during which the formation of diphenylurea precipitate was visible. Afterwards keto-alkyne **38** (0.6 g, 2.19 mmol) was added and the mixture was left stirring for 18 h. After cooling, the reaction was quenched by adding a few drops of water, and stirring was continued for 1 h. The reaction mixture was filtered, dried over anhydrous Na_2SO_4 and toluene was removed under reduced pressure. The crude mixture was subjected to flash column chromatography (hexane 100% towards hexane/ethyl acetate 1:1) to obtain compound **39** in moderate yield. (0.35 g, 41%) Yellow oil; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.41 (s, 9H, Boc), 3.01-3.33 (m, 2H, CH_2Ph), 5.25 (s br, 1H, CH), 5.35 (s br, 1H, NH), 3.43 (s, 1H, HCC), 4.68 (m, 1H, CH), 4.99 (s br, 1H, NH), 7.48 (s, 1H, isoxazole-H), 7.03-7.83 (m, 10H, $2 \times \text{Ph}$); MS (ESI) m/z : 415 [$M+\text{Na}$] $^+$.

(S)-tert-Butyl 1-oxo-3-phenyl-1-(1-phenyl-1H-1,2,3-triazol-4-yl)propan-2-ylcarbamate (40): In a 250 mL round-bottomed flask was **38** (2.14 g, 7.83 mmol) dissolved in methanol to give a yellowish solution. Copper(I)iodide (1.49 g, 7.83 mmol) was added. Azidobenzene (as a solution in diethyl ether) was added to the solution and left stirring over the weekend (60 h) at room temperature. The resulting greenish mixture was filtered through celite and subjected to column chromatography (hexane/ethyl acetate 3:1). The triazine product **40** was isolated in low yield as a syrup. (0.33 g, 11%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.41 (s, 9H,

Boc), 3.18-3.45 (m, 2H, CH₂Ph), 5.37 (d, $J=6.4$ Hz, 1H, NH), 5.65 (m, 1H, CH), 7.17-7.78 (m, 10H, Ph), 8.57 (s, 1H, triazole-H); MS (ESI) m/z : 415 [$M+Na$]⁺.

N-[(S)-1-Oxo-1-[(S)-1-oxo-3-phenyl-1-(3-phenylisoxazol-5-yl)propan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (6): In a 100 mL round-bottomed flask isoxazole **39** (0.35 g, 0.9 mmol) was dissolved in CH₂Cl₂ (3 mL) to give a yellow solution. Upon addition of trifluoroacetic acid (3 mL) the solution turned darker brown. The mixture was left stirring at room temperature for 2 h, and then the residue was dried under reduced pressure. The deprotected compound was detected using mass spectroscopy. Mu-Phe-OH (0.25 g, 0.9 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (0.37 g, 1.96 mmol) and hydroxybenzotriazole (0.15 g, 0.98 mmol) are dissolved in CH₂Cl₂ (20 mL) and left stirring at room temperature. Triethylamine (0.38 mL, 2.68 mmol) is added and the mixture is stirred for 30 min., after which a CH₂Cl₂ solution of deprotected isoxazole **39** is added dropwise and stirring was continued overnight (15 h). A fivefold volume of water was added and work-up was continued following the procedure as described for **28** yielding a mixture of two diastereomers, ratio 2:1 (0.18 g, 38%). Yellow oil; HPLC ($\lambda=214$ nm): $t_R=25.56$ min (broad signal); ¹H-NMR (400 MHz, CDCl₃): $\delta=2.97$ -3.19 (m, 6H, 2 $\times$ CH₂Ph), 3.19-3.34 (m, 6H, CH₂N), 3.56-3.64 (m, 6H, CH₂O), 4.64 (m, 1.5H, CH), 5.05 (m, 1.5H, NH), 5.46 (q, $J=6.8$ Hz, 0.5H, CH), 5.53 (q, $J=6.8$ Hz, 1H, CH), 6.98-7.84 (m, 22H, Ph), 7.49 (s, 0.5H, isoxazole-H), 7.51 (s, 1H, isoxazole-H); MS (ESI) m/z : 575 [$M+Na$]⁺; LC-MS: $t_R=18.3$ -18.5 min, m/z : 553 [$M+H$]⁺.

N-[(S)-1-Oxo-1-[(S)-1-oxo-3-phenyl-1-(1-phenyl-1H-1,2,3-triazol-4-yl)propan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (7): In a 50 mL round-bottomed flask triazole **40** (0.25 g, 0.66 mmol) was deprotected and and coupled using a mixture of Mu-Phe-OH (0.18 g, 0.66 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.14 g, 0.73 mmol), hydroxybenzotriazole (0.1 g, 0.73 mmol) and triethylamine (0.28 mL, 1.99 mmol) following the procedure as described for **6**, furnishing a mixture of two diastereomers, ratio 1:1 (0.12 g, 32.6 %). Using preparative TLC these isomers were separated in isomers **7a** and **7b**. **Isomer 7a**: White solid; HPLC ($\lambda=254$ nm) $t_R=23.42$ min; ¹H-NMR (400 MHz, CDCl₃): $\delta=2.97$ -3.15 (m, 4H, 2 $\times$ CH₂Ph), 3.29 (m, 4H, CH₂N), 3.63 (t, $J=4.8$ Hz, 4H, CH₂O), 4.65 (m, 1H, CH), 5.03 (d, $J=7.2$ Hz, 1H, NH), 5.79 (m, 1H, CH), 6.76 (d, $J=6.8$ Hz, 1H, NH), 6.96-7.84 (m, 15H, 3 $\times$ Ph), 8.61 (s, 1H, triazole-H); MS (ESI) m/z : 415 [$M+Na$]⁺; LC-MS: $t_R=16.8$ min, m/z : 553 [$M+H$]⁺. **Isomer 7b**: White solid; HPLC ($\lambda=254$ nm): $t_R=23.73$ min; ¹H-NMR (400 MHz, CDCl₃): $\delta=3.04$ (m, 2H, CH₂Ph), 3.20-3.35 (m, 6H, CH₂N + CH₂Ph), 3.62 (m, 4H, CH₂O), 4.66 (m, 1H, CH), 5.00 (d, $J=6.8$ Hz, 1H, NH), 5.84 (m, 1H, CH), 6.81 (d, $J=7.2$ Hz, 1H, NH), 6.99-7.77 (m, 15H, 3 $\times$ Ph), 8.52 (s, 1H, triazole-H); MS (ESI) m/z : 415 [$M+Na$]⁺; LC-MS $t_R=16.9$ min, m/z : 553 [$M+H$]⁺.

(S)-tert-Butyl 1-[1-(benzyloxymethyl)-1H-1,2,3-triazol-4-yl]-1-oxo-3-phenylpropan-2-ylcarbamate (41): In a 250 mL round-bottomed flask equipped with a reflux cooler was (*S*)-*tert*-butyl 3-oxo-1-phenylpent-4-yn-2-ylcarbamate **38** (1.56 g, 5.71 mmol) dissolved in methanol (30 mL) to give a yellow solution. Copper(I)iodide (2.17 g, 11.41 mmol) and [(azidomethoxy)methyl]benzene (1.21 g, 7.42 mmol) were added and the resulting suspension was stirred for 5 h at reflux conditions and at room temperature for 12 h. After evaporation of methanol, the crude mixture is directly loaded onto a silica gel column using a mixture of hexane and ethyl acetate (gradually going to 100% ethyl acetate) as eluent. (1.7 g, 71%) Syrup; ¹H-NMR (400 MHz, CDCl₃): $\delta=1.41$ (s, 9H, Boc), 3.15 (m, 1H, CH₂Ph), 3.41 (m, 1H, CH₂Ph), 4.61 (s, 2H, OCH₂Ph), 5.29 (s br, 1H, CH), 5.97 (s br, 1H, NH), 5.78 (s, 2H, NCH₂O), 7.13-7.44 (m, 10H, 2 $\times$ Ph), 8.27 (s, 1H, triazole-H); MS (ESI) m/z : 459 [$M+Na$]⁺.

tert-Butyl (2S)-1-[1-(benzyloxymethyl)-1H-1,2,3-triazol-4-yl]-1-hydroxy-3-

phenylpropan-2-ylcarbamate (42): In a 250 mL round-bottomed flask was **41** (0.91 g, 2.09 mmol) dissolved in methanol (20 mL) to give a yellow solution which was cooled to -20°C . Sodium borohydride (0.04 g, 1.04 mmol) was added and the reaction mixture was stirred for 1 h. After 1 h, the reaction was quenched by the addition of acetone, and the mixture was stirred for 15 min and concentrated in vacuo. The residue was dissolved in water (100 mL), extracted with ethyl acetate (3×60 mL) and the combined extracts were washed with brine (2×50 mL), dried over anhydrous Na_2SO_4 , filtered and concentrated in vacuo to yield **42** as a mixture of two diastereomers. (0.87 g, 95%) Oil; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.33 (s, 9H, Boc), 1.37 (s, 9H, Boc), 2.89-3.06 (m, 4H, $2 \times \text{CH}_2\text{Ph}$), 4.12 (m, 1H, CH), 4.28 (m, 1H, CH), 4.51 (s, 1H, OCH_2Ph), 4.59 (s, 1H, OCH_2Ph), 4.98 (m, 1H, CH), 5.07 (m, 1H, CH), 5.67 (s, 2H, NCH_2O), 5.74 (s, 2H, NCH_2O), 7.16-7.37 (m, 20H, Ph), 7.76 (s br, 2H, $2 \times$ triazole-H); MS (ESI) m/z : 461 $[\text{M}+\text{Na}]^+$.

N-((2S)-1-((2S)-1-[1-(Benzyloxymethyl)-1H-1,2,3-triazol-4-yl]-1-hydroxy-3-

phenylpropan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)morpholine-4-carboxamide (43): Compound **42** (0.58 g, 1.94 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **43**. Two clear diastereomers were visible in $^1\text{H-NMR}$ due to the alcohol. (0.42 g, 36%) Oil; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.65-3.11 (m, 8H, $4 \times \text{CH}_2\text{Ph}$), 3.13-3.33 (m, 8H, $2 \times \text{CH}_2\text{N}$), 3.53-3.65 (m, 8H, $2 \times \text{CH}_2\text{O}$), 4.39 (m, 2H, $2 \times \text{CH}$), 4.53 (s, 1H, OCH_2Ph), 4.57 (s, 1H, OCH_2Ph), 4.76 (m, 2H, $2 \times \text{CH}$), 4.79 (d, $J=3.2\text{ Hz}$, 1H, CHOH), 4.92 (d, $J=3.2\text{ Hz}$, 1H, CHOH), 5.67 (s, 2H, NCH_2O), 5.72 (s, 2H, NCH_2O), 7.06-7.38 (m, $2 \times 15\text{H}$, Ph), 7.62 (s 1H, triazole-H), 7.17 (s, 1H, triazole-H); MS (ESI) m/z : 621 $[\text{M}+\text{Na}]^+$.

N-((S)-1-((S)-1-[1-(Benzyloxymethyl)-1H-1,2,3-triazol-4-yl]-1-oxo-3-phenylpropan-2-

ylamino)-1-oxo-3-phenylpropan-2-yl)morpholine-4-carboxamide (8): Compound **43** (0.42 g, 0.7 mmol) was used in procedure B and yielded keto-triazole **8** (0.22 g, 53%) Off-white solid; HPLC ($\lambda=214\text{ nm}$): $t_R=23.66\text{ min}$; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.97-3.12 (m, 2H, CH_2Ph), 3.14 (m, 1H, CH_2Ph), 3.27 (m, 4H, CH_2N), 3.33-3.38 (m, 1H, CH_2Ph), 3.63 (m, 4H, CH_2O), 4.59 (s, 2H, OCH_2Ph), 5.04 (d, $J=7.2\text{ Hz}$, 1H, NH), 5.76 (s, 2H, NCH_2O), 6.70 (d, $J=7.2\text{ Hz}$, 1H, NH), 7.15-7.43 (m, 15H, Ph), 8.30 (s, 1H, triazole-H); MS (ESI) m/z : 619 $[\text{M}+\text{Na}]^+$; LC-MS: $t_R=16.6\text{ min}$, m/z 597 $[\text{M}+\text{H}]^+$.

N-((S)-1-Oxo-1-((S)-1-oxo-3-phenyl-1-(1H-1,2,3-triazol-4-yl)propan-2-ylamino)-3-

phenylpropan-2-yl)morpholine-4-carboxamide (9): To a stirred solution of **8** (0.3 g, 0.503 mmol) in ethanol (10 mL), palladium on carbon (0.535 g, 5.03 mmol) was added. The resulting reaction mixture was put under hydrogen atmosphere (excess) and stirred overnight at room temperature. The reaction time was prolonged to 5 days because TLC still showed starting product. After 5 days of stirring at room temperature the reaction was filtered through a plug of celite and subjected to flash column chromatography (hexane/ethyl acetate 1:4 system) to yield **9** in moderate yield. (70 mg, 30%) Solid; HPLC ($\lambda=214\text{ nm}$): $t_R=17.29\text{ min}$; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =3.04-3.12 (m, 2H, CH_2Ph), 3.16 (m, 1H, CH_2Ph), 3.30 (m, 4H, CH_2N), 3.33-3.38 (m, 1H, CH_2Ph), 3.63 (m, 4H, CH_2O), 4.67 (s, 1H, CH), 5.08 (d, $J=4.8\text{ Hz}$, 1H, NH), 5.81 (s, 1H, CH), 6.91-7.26 (m, 10H, Ph), 8.17 (s, 1H, triazole-H); MS (ESI) m/z : 499 $[\text{M}+\text{Na}]^+$; LC-MS $t_R=13.2\text{ min}$, m/z 477 $[\text{M}+\text{H}]^+$.

N-Formyl-N-(2-oxo-2-phenylethyl)formamide (45): A mixture of sodium diformylamide (5 g, 52.6 mmol) and 2-bromoacetophenone (8.7 g, 43.8 mmol) in acetonitrile (60 mL) is stirred at room temperature for 2 h and then heated to 70°C . The hot mixture is filtered and the solid is washed with hot acetonitrile ($2 \times 25\text{ mL}$). The combined organic filtrates are evaporated to 30 mL and allowed to stand undisturbed for thorough crystallization. The crystals are collected by filtration and washed with chloroform (10 mL) to give **45** in high

yield. (4.78 g, 99%); $^1\text{H-NMR}$ (400 MHz, MeOD): δ =4.78 (s, 2H, CH₂), 7.51-7.66 (m, 3H, Ph), 8.00-8.03 (m, 2H, Ph), 8.09 (s, 1H, CHO), 8.23 (s, 1H, CHO); MS (ESI) m/z : 185 [M +Na]⁺ and 349 [$2M$ +Na]⁺, the monoformyl product is visible in MS.

5-Phenylthiazole (46): The bisformamide derivative **45** (3 g, 15.7 mmol) was dissolved in chloroform (70 mL). Then, triethylamine (4.4 mL, 31.4 mmol) was added to the stirred mixture, followed by phosphorous pentasulfide (13.9 g, 31.4 mmol). The mixture was stirred at 60°C for the appropriate time (40-60 min). After cooling to room temperature, water (80 mL) was added and the mixture was stirred for an additional hour. Dichloromethane (60 mL) was then added and the resulting layers were separated. The organic phase was washed with water and brine, dried over anhydrous Na₂SO₄ and finally the solvent was removed in vacuo. A dark-brown to black oil was obtained which was loaded onto a silica gel column using toluene; because of the limited solubility of the product in toluene a soxhlet was utilized to extract all crude thiazole out of the mixture. A mixture of hexane and ethyl acetate (1:1 towards 1:3) was used as eluent furnishing compound **46** as a dark yellow solid. (0.98 g, 39%); $^1\text{H-NMR}$ (400 MHz, CDCl₃): δ =7.33-7.60 (m, 5H, Ph), 8.09 (s, 1H, CHN), 8.76 (s, 1H, SCHN); MS (ESI) m/z : 162 [M +H]⁺.

(S)-tert-Butyl 1-oxo-3-phenyl-1-(thiazol-2-yl)propan-2-ylcarbamate (47): To thiazole (2.42 mL, 33.76 mmol) and TMEDA (5.1 mL, 33.76 mmol) in THF (40 mL) at -78°C was added *n*BuLi in hexane (21.08 mL, 33.76 mmol, 1.6 M) dropwise over a period of 10 min, so that the internal temperature is controlled. After addition the solution is stirred for 15 min at -78°C. Weinreb amide **37** (2.6 g, 8.43 mmol) was dissolved in dry THF (40 mL), cooled with a dry ice/acetone bath, and then added via cannula to the lithiothiazole solution at -78°C, where the reaction stirred for 2 h. The reaction was quenched by pouring into a saturated aqueous ammonium chloride solution (600 mL) and shaking vigorously. The aqueous layer was extracted with ethyl acetate (2 × 500 mL), and the combined organic layers were washed with brine (400 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified on a silica gel column, eluting with ethyl acetate/hexane (50% v/v), and then EtOAc (100%), to afford the title compound (0.85 g, 30%) as a white foam; HPLC (λ =214 nm): t_R =25.86 min; $^1\text{H-NMR}$ (400 MHz, CDCl₃): δ =1.40 (s, 9H, Boc), 3.14 (m, 1H, CH₂Ph), 3.38 (m, 1H, CH₂Ph), 5.29 (s br, 1H, CH), 5.73 (s br, 1H, NH), 7.11 (m, 2H, Ph), 7.19-7.27 (m, 3H, Ph), 7.71 (d, J =2.8 Hz, 1H, thiazole H₄), 8.06 (d, J =2.8 Hz, 1H, thiazole H₅); $^{13}\text{C-NMR}$ (100 MHz, CDCl₃): δ =28.23 (Boc), 38.65 (CH₂Ph), 57.47 (CH), 79.75 (Boc), 126.66, 126.85, 128.39, 129.40 (Ph), 136.03 (thiazole C₄), 145.11 (thiazole C₅), 155.03 (Boc), 164.70 (thiazole C₂), 191.36 (Het-C=O) ppm.

(S)-tert-Butyl 1-(benzo[d]thiazol-2-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (48): To a cooled solution of the benzo[d]thiazole (1.597 mL, 14.53 mmol) in dry THF at -78°C, a solution of *n*BuLi (1.6M in hexane, 9.08 mL, 14.53 mmol) was slowly added. After 30 min, a solution of Weinreb amide **37** in dry THF was slowly added and the reaction was stirred at -78°C for 2 h. The reaction mixture was quenched with 1N HCl, and extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried over anhydrous Na₂SO₄, filtered and evaporated in vacuo. The crude mixture was then purified using flash chromatography (eluent system: hexane 100% towards hexane/ethyl acetate 70:30 in 45 min) furnishing **48** as a yellowish oil. (1.2 g, 86%); $^1\text{H-NMR}$ (400 MHz, CDCl₃): δ =1.42 (s, 9H, Boc), 3.23 (m, 1H, CH₂Ph), 3.46 (m, 1H, CH₂Ph), 5.31 (s br, 1H, CH), 5.90 (s br, 1H, NH), 7.13-7.29 (m, 5H, Ph), 7.55 (m, 2H, benzothiazole H₅, H₆), 8.01 (d, J =7.6 Hz, 1H, benzothiazole H₇), 8.24 (d, J =8.0 Hz, 1H, benzothiazole H₄); MS (ESI): m/z 405 [M +Na]⁺.

(S)-tert-Butyl 1-oxo-3-phenyl-1-(5-phenylthiazol-2-yl)propan-2-ylcarbamate (49): In a 100 mL round-bottomed flask 5-phenylthiazole **46** (0.77 g, 4.78 mmol) was dissolved in dry

THF (10 mL). The solution was kept under nitrogen atmosphere and cooled to -78°C using a dry ice/acetone bath. Lithium diisopropylamide (3.1 mL, 6.2 mmol) was added dropwise via syringe and the mixture was left stirring at this temperature for 30 min. A pre-cooled solution of Weinreb amide **37** (1.47 g, 4.78 mmol) in dry THF (15 mL) was added dropwise via cannula over a period of 10 min and the mixture is stirred at -78°C for 2 h. The solution is allowed to heat to room temperature and is quenched by adding water (75 mL), and extracted with ethyl acetate (3×40 mL). The combined organic phases are washed with 1N HCl (50 mL), water (50 mL), sat. NaHCO_3 (50 mL) and brine (50 mL). After drying over anhydrous Na_2SO_4 , the solution was filtered and concentrated by rotary evaporation. This crude mixture was then subjected to flash chromatography (hexane 100% towards hexane/ethyl acetate 1:1 in 35 min) (0.75 g, 38%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.43$ (s, 9H, Boc), 3.08-3.17 (m, 2H, CH_2), 5.30 (s, 1H, CH), 5.74 (s, 1H, NH), 7.13-7.67 (m, 10H, $2 \times$ Ph), 8.21 (s, 1H, thiazole-H); MS (ESI) m/z : 431 $[\text{M}+\text{Na}]^+$.

tert-Butyl (2S)-1-hydroxy-3-phenyl-1-(thiazol-2-yl)propan-2-ylcarbamate (50): In a 50 mL round-bottomed flask **47** (0.8 g, 2.40 mmol) dissolved in methanol (10 mL) to give a yellow solution, which was cooled to 0°C using an ice bath and magnetically stirred under nitrogen atmosphere. Sodium borohydride (0.11 g, 2.96 mmol) was added and the mixture was stirred for 2 h allowing temperature to raise to room temperature. The reaction mixture was slowly quenched with 1N HCl, and extracted with EtOAc (3×50 mL). The combined organic layers were washed with 1N HCl, brine and dried over anhydrous Na_2SO_4 , filtered and concentrated in vacuo. The crude residue (a mixture of diastereomers ratio 1: 0.8) was used as such without further purification for the following synthetic procedure (0.79 g, 98%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.31$ (s, 9H, Boc), 1.33 (s, 7H, Boc), 2.82-3.11 (m, 4H, CH_2Ph), 4.18 (m, 0.8H, CH), 4.29 (m, 1H, CH), 5.05 (s br, 0.8H, CHOH), 5.12 (s br, 1H, CHOH), 5.35 (d, $J=7.6$ Hz, 1H, NH), 7.16-7.25 (m, 10H, Ph), 7.26 (d, $J=2.8$ Hz, 1H, thiazole H_5), 7.30 (d, $J=2.8$ Hz, 0.8H, thiazole H_5), 7.68 (s br, 0.8H, thiazole H_4), 7.75 (s br, 1H, thiazole H_4); MS (ESI) m/z : 357 $[\text{M}+\text{Na}]^+$.

tert-Butyl (2S)-1-(benzo[d]thiazol-2-yl)-1-hydroxy-3-phenylpropan-2-ylcarbamate (51): Compound **48** (0.67 g, 1.75 mmol) and sodium borohydride (0.082 g, 2.16 mmol) were reacted as described for compound **50**. The crude residue (a mixture of diastereomers ratio 1: 0.6) was used as such without further purification for the following synthetic procedure (0.67 g, quant.). $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.31$ (s, 6H, Boc), 1.38 (s, 9H, Boc), 3.02 (d, $J=6.4$ Hz, 2H, CH_2Ph), 3.16 (d, $J=6.0$ Hz, 1.4H, CH_2Ph), 4.25 (m, 0.7H, CH), 4.37 (m, 1H, CH), 5.13 (s br, 1H, CHOH), 5.25 (s br, 0.7H, CHOH), 7.18-7.53 (m, 12H, Ph), 7.87-8.05 (m, 3.5H, Ph); MS (ESI) m/z : 407 $[\text{M}+\text{Na}]^+$.

N-((2S)-1-((2S)-1-Hydroxy-3-phenyl-1-(thiazol-2-yl)propan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)morpholine-4-carboxamide (52): Compound **50** (0.79 g, 2.36 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **52**. (0.18 g, 15%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=2.75$ -3.03 (m, 6H, $2 \times \text{CH}_2\text{Ph}$), 3.07-3.26 (m, 6H, CH_2N), 3.53-3.58 (m, 6H, CH_2O), 4.43 (m, 1H, CH), 4.50 (m, 1H, CH), 4.66 (m, 1H, CH), 4.90 (d, $J=6.8$ Hz, 1H, NH), 4.95-5.02 (m, 1.5H, CHOH), 6.99 (d, $J=7.2$ Hz, 1H, NH), 7.06-7.27 (m, 15H, Ph), 7.32 (d, $J=3.2$ Hz, 1H, thiazole H_4), 7.67 (d, $J=3.2$ Hz, 0.5H, thiazole H_5), 7.75 (d, $J=3.2$ Hz, 1H, thiazole H_5); MS (ESI) m/z : 517 $[\text{M}+\text{Na}]^+$.

N-((2S)-1-((2S)-1-(Benzo[d]thiazol-2-yl)-1-hydroxy-3-phenylpropan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)morpholine-4-carboxamide (53): Compound **51** (0.67 g, 1.75 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **53**. (0.31 g, 33%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=2.79$ -3.05 (m, 6H, $2 \times \text{CH}_2\text{Ph}$), 3.06-3.21 (m, 6H, CH_2N), 3.47-3.57 (m, 6H, CH_2O), 4.41 (m, 1H, CH), 4.46 (m, 0.5H, CH), 4.49 (m, 0.5H,

CH), 4.70 (m, 1H, CH), 4.78 (d, $J=6.4$ Hz, 0.5H, NH), 4.94 (d, $J=6.4$ Hz, 0.5H, NH), 5.06 (s br, 0.5H, CHOH), 5.09 (s br, 1H, CHOH), 6.83 (d, $J=8.0$ Hz, 1H, NH), 6.92-7.27 (m, 15H, Ph), 7.34-7.52 (m, 3H, benzothiazole H₅, H₆), 7.84-7.99 (m, 3H, benzothiazole H₄, H₇); MS (ESI) m/z : 567 [$M+Na$]⁺.

N-[(S)-1-Oxo-1-[(S)-1-oxo-3-phenyl-1-(thiazol-2-yl)propan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (10): Compound **52** (0.18 g, 0.364 mmol) was used in procedure B and yielded keto-thiazole **10** as a white solid. (0.1 g, 56%); HPLC ($\lambda=214$ nm) $t_R=21.22$ min; ¹H-NMR (400 MHz, CDCl₃): $\delta=2.96$ -3.13 (m, 3H, CH₂Ph), 3.25 (m, 6H, CH₂N), 3.31-3.33 (m, 1H, CH₂Ph), 3.61 (m, 6H, CH₂O), 4.60 (m, 1H, CH), 5.20 (d, $J=7.2$ Hz, 1H, NH), 5.83 (m, 1H, CH), 6.80 (d, $J=7.2$ Hz, 1H, NH), 6.94 (t, $J=3.6$ Hz, 2H, Ph), 7.14-7.24 (m, 8H, Ph), 7.70 (d, $J=3.2$ Hz, 1H, thiazole H₄), 8.03 (d, $J=3.2$ Hz, 1H, thiazole H₅); ¹³C-NMR (100 MHz, CDCl₃): $\delta=38.19$ (CH₂Ph), 38.61 (CH₂Ph), 43.90 (CH₂NCH₂), 55.46 (CH), 56.47 (CH), 66.31 (CH₂OCH₂), 126.75, 126.790 126.84, 128.330 128.44, 129.24, 129.38, 135.64 (Ph), 136.86 (thiazole C₄), 145.05 (thiazole C₅), 156.82 (C=O urea), 164.54 (thiazole C₂), 171.31 (C=O amide), 189.96 (Het-C=O) ppm; MS (ESI) m/z : 515 [$M+Na$]⁺.

N-[(S)-1-[(S)-1-(Benzo[d]thiazol-2-yl)-1-oxo-3-phenylpropan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (11): Compound **53** (0.29 g, 0.532 mmol) was used in procedure B and yielded keto-benzothiazole **11** as a white solid. (105 mg, 36%); HPLC ($\lambda=214$ nm): $t_R=25.44$ min; ¹H-NMR (400 MHz, CDCl₃): $\delta=2.94$ -3.17 (m, 3H, CH₂Ph), 3.27 (m, 4H, CH₂N), 3.35-3.40 (m, 4H, CH₂Ph), 3.63 (m, 6H, CH₂O), 4.59 (m, 1H, CH), 5.07 (s br, 1H, NH), 5.92 (m, 1H, CH), 6.62 (s br, 1H, NH), 6.93 (m, 2H, Ph), 7.09-7.22 (m, 8H, Ph), 7.55-7.64 (m, 2H, benzothiazole H₅, H₆), 8.01 (m, 1H, benzothiazole H₇), 8.21 (m, 1H, benzothiazole H₄); ¹³C-NMR (100 MHz, CDCl₃): $\delta=38.31$ (CH₂Ph), 38.76 (CH₂Ph), 43.94 (CH₂NCH₂), 55.59 (CH), 56.69 (CH), 66.36 (CH₂OCH₂), 122.35, 125.81, 126.85, 126.96, 127.16, 128.05, 128.43, 128.55, 129.33, 129.39 (Ph + benzothiazole), 135.56 (benzothiazole bridged C), 136.84 (Ph), 137.1 (Ph), 153.45 (benzothiazole bridged C), 156.82 (C=O urea), 163.76 (benzothiazole C₂), 171.24 (C=O amide), 191.46 (Het-C=O) ppm.

N-[(S)-1-Oxo-1-[(S)-1-oxo-3-phenyl-1-(5-phenylthiazol-2-yl)propan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (12): In a 50 mL round-bottomed flask thiazole **49** (0.51 g, 1.25 mmol) was deprotected and coupled using a mixture of Mu-Phe-OH (0.35 g, 1.25 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.53 g, 2.75 mmol), hydroxybenzotriazole (0.21 g, 1.37 mmol) and triethylamine (0.53 mL, 3.75 mmol) following the procedure as described for **6**, furnishing a mixture of two diastereomers, ratio 2:1 (0.18 g, 54%); HPLC ($\lambda=214$ nm) $t_{R1}=24.34$ min, $t_{R2}=24.56$ min (ratio 34:66); ¹H-NMR (400 MHz, CDCl₃): $\delta=2.97$ -3.22 (m, 6H, 2 × CH₂Ph), 3.27 (m, 6H, CH₂N), 3.61 (m, 6H, CH₂O), 4.59 (m, 0.5H, CH), 4.65 (m, 1H, CH), 5.08 (d, $J=7.2$ Hz, 1H, NH), 5.12 (d, $J=7.2$ Hz, 0.5H, NH), 5.83 (m, 0.5H, CH), 5.93 (m, 1H, CH), 6.68 (d, $J=7.2$ Hz, 0.5H, NH), 6.89 (d, $J=7.2$ Hz, 0.5H, NH), 6.95-7.66 (m, 22H, Ph), 8.16 (s, 0.5H, thiazole-H), 8.17 (s, 1H, thiazole-H); MS (ESI) m/z : 591 [$M+Na$]⁺; LC-MS: $t_R=17.7$ -17.9 min, m/z : 569 [$M+H$]⁺.

tert-Butyl (2S)-1-hydroxy-3-phenyl-1-(5-phenyloxazol-2-yl)propan-2-ylcarbamate (54): To a 250 mL round-bottomed flask was added THF (7 mL) under N_{2(g)}. After the solution was cooled to an internal temperature of -15°C, 5-phenyloxazole (1.3 g, 8.96 mmol) was added and a dropping funnel was used to control the addition of isopropylmagnesium chloride (4.48 mL, 8.96 mmol) over 5 min, maintaining a temperature of below -10°C. The homogeneous solution was then stirred for 20 min. After this period, compound **18** (0.744 g,

2.99 mmol) was added in THF (10 mL) over 5 min maintaining an internal temperature below -14°C . The reaction was left stirring for 30 min and was then warmed to a jacket temperature of 28°C and stirred for 4 h. After this hold time, the reaction was quenched by addition of distilled water (150 mL). The resulting yellow white suspension was extracted 3 times with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4 , filtered, and concentrated to a crude mass of 0.85 g. The material was purified column chromatography using hexane – ethyl acetate 1:1 as eluent to provide 0.21 g of product **54**. (0.21 g, 19%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.30 (s, 9H, Boc), 1.32 (s, 5H, Boc), 2.80-2.98 (m, 3H, $1.5 \times \text{CH}_2\text{Ph}$), 4.27 (m, 1H, CH), 4.33 (m, 0.5H, CH), 4.75 (d, J =3.2 Hz, 1H, CHOH), 4.83 (d, J =3.6 Hz, 0.6H, CHOH), 4.96 (d, J =9.6 Hz, 1H, NH), 7.03-7.56 (m, 11H, $2 \times \text{Ph} + \text{oxazole-H}$); LC-MS: t_{R} =17.7-17.9 min, m/z : 395 $[\text{M}+\text{H}]^+$.

tert-Butyl (2S)-1-(benzo[d]oxazol-2-yl)-1-hydroxy-3-phenylpropan-2-ylcarbamate (55): Benzo[d]oxazole (0.26 g, 2.17 mmol), isopropylmagnesium bromide (1.08 mL, 2.17 mmol) and compound **18** (0.18 g, 0.722 mmol) were reacted following the procedure as described for **54**. (0.17 g, 64%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =1.30 (s, 9H, Boc), 1.34 (s, 9H, Boc), 2.75-3.00 (m, 4H, $2 \times \text{CH}_2\text{Ph}$), 4.30 (m, 1H, CH), 4.39 (m, 0.8H, CH), 4.92-4.98 (m, 2H, $2 \times \text{CHOH}$), 7.05-7.66 (m, 18H, $2 \times \text{Ph} + \text{benzoxazole-H}$); MS (ESI) m/z : 369 $[\text{M}+\text{H}]^+$.

N-[(2S)-1-[(2S)-1-Hydroxy-3-phenyl-1-(5-phenyloxazol-2-yl)propan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (56): Compound **54** (0.19 g, 0.482 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **56**. (0.26 g, 82%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.75-3.08 (m, 8H, $2 \times (2 \times \text{CH}_2\text{Ph})$), 3.09-3.30 (m, 8H, $2 \times \text{CH}_2\text{NCH}_2$), 3.52-3.62 (m, 8H, $2 \times \text{CH}_2\text{OCH}_2$), 4.41 (m, 1H, CH), 4.50 (m, 1H, CH), 4.59 (m, 2H, $2 \times \text{CH}$), 4.70 (m, 1H, CH), 4.75 (d, J =3.6 Hz, 1H, CHOH), 4.86 (d, J =3.6 Hz, 1H, CHOH), 4.92-4.97 (m, 2H, $2 \times \text{NH}$), 6.93 (d, J =7.2 Hz, 1H, NH), 6.99-7.64 (m, 32H, $2 \times (3 \times \text{Ph} + \text{oxazole-H})$); MS (ESI) m/z : 577 $[\text{M}+\text{Na}]^+$.

N-[(2S)-1-[(2S)-1-(Benzo[d]oxazol-2-yl)-1-hydroxy-3-phenylpropan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (57): Compound **55** (0.15 g, 0.407 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **57** as a mixture of two diastereomers (0.11 g, 51%); HPLC (λ =214 nm): $t_{\text{R}1}$ =20.24 min, $t_{\text{R}2}$ =20.33 min (ratio 40:60); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.76-3.10 (m, 8H, $2 \times (2 \times \text{CH}_2\text{Ph})$), 3.10-3.25 (m, 8H, $2 \times \text{CH}_2\text{NCH}_2$), 3.54-3.58 (m, 8H, $2 \times \text{CH}_2\text{OCH}_2$), 4.43 (m, 1H, CH), 4.49 (m, 1H, CH), 4.64 (m, 2H, $2 \times \text{CH}$), 4.83 (m, 1H, CH), 4.90 (m, 1H, CHOH), 4.94 (m, 1H, CHOH), 4.98-5.08 (m, 2H, $2 \times \text{NH}$), 6.93-7.39 (m, 24H, $2 \times (2 \times \text{Ph} + \text{benzoxazole-H}_4 \text{ and } -\text{H}_7)$), 7.47-7.71 (m, 4H, $2 \times \text{benzoxazole-H}_5 \text{ and } -\text{H}_6$); MS (ESI) m/z : 551 $[\text{M}+\text{Na}]^+$.

N-[(S)-1-Oxo-1-[(S)-1-oxo-3-phenyl-1-(5-phenyloxazol-2-yl)propan-2-ylamino]-3-phenylpropan-2-yl]morpholine-4-carboxamide (13): Compound **56** (0.22 g, 0.397 mmol) was used in procedure B and yielded keto-oxazole **13** as a white solid. (85 mg, 40%); HPLC (λ =214 nm): $t_{\text{R}1}$ =24.26 min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ =2.94-3.26 (m, 4H, $2 \times \text{CH}_2\text{Ph}$), 3.27 (m, 4H, CH_2NCH_2), 3.62 (m, 4H, CH_2OCH_2), 4.58 (m, 1H, CH), 5.08 (d, J =7.2 Hz, 1H, NH), 5.68 (m, 1H, CH), 6.62 (d, J =7.2 Hz, 1H, NH), 6.97 (m, 2H, Ph), 7.13-7.26 (m, 7H, Ph), 7.42-7.50 (m, 4H, Ph), 7.52 (s, 1H, oxazole-H), 7.77 (m, 2H, Ph); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ =38.35 (CH_2Ph), 38.78 (CH_2Ph), 43.99 (CH_2NCH_2), 55.53 (CH), 56.62 (CH), 66.42 (CH_2OCH_2), 124.16, 125.50, 126.46, 126.97, 127.06, 128.56, 128.63, 129.22, 129.32, 129.52, 130.30, 135.49, 136.90 (Ph + oxazole C_4), 154.77 (oxazole C_5), 155.51 (oxazole C_2), 156.87 (C=O urea), 171.33 (C=O amide), 184.53 (Het-C=O) ppm; MS (ESI) m/z : 575 $[\text{M}+\text{Na}]^+$.

***N*-{[(*S*)-1-[(*S*)-1-(Benzo[d]oxazol-2-yl)-1-oxo-3-phenylpropan-2-ylamino]-1-oxo-3-phenylpropan-2-yl}morpholine-4-carboxamide (**14**):** Compound **57** (0.1 g, 0.189 mmol) was used in procedure B and yielded keto-oxazole **14** as an off-white solid (104 mg, 55%); HPLC ($\lambda=214$ nm): $t_{R1}=23.07$ min; $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=2.94\text{--}3.37$ (m, 4H, $2 \times \text{CH}_2\text{Ph}$), 3.27 (m, 4H, CH_2NCH_2), 3.61 (m, 4H, CH_2OCH_2), 4.59 (m, 1H, CH), 5.12 (d, $J=7.2$ Hz, 1H, NH), 5.77 (m, 1H, CH), 6.75 (d, $J=7.2$ Hz, 1H, NH), 6.99–7.26 (m, 10H, Ph), 7.48 (m, 1H, benzoxazole- H_5), 7.56 (m, 1H, benzoxazole- H_6), 7.66 (m, 1H, benzoxazole- H_4), 7.89 (m, 1H, benzoxazole- H_7); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): $\delta=38.11$ (CH_2Ph), 38.77 (CH_2Ph), 43.99 (CH_2NCH_2), 55.58 (CH), 57.12 (CH), 66.41 (CH_2OCH_2), 111.99 (benzoxazole C_4), 122.59 (benzoxazole C_7), 126.01, 126.91, 127.14, 128.59, 128.69, 128.97, 129.33, 129.37, 135.28, 136.81, 140.47 (Ph + benzoxazole C_5 , C_6 , C_{7a}), 150.69 (benzoxazole C_2), 155.65 (benzoxazole C_{3a}), 156.91 (C=O urea), 171.53 (C=O amide), 186.83 (Het-C=O) ppm; MS (ESI) m/z : 549 [$M+\text{Na}$] $^+$.

***tert*-Butyl (2*S*)-1-cyano-3-phenyl-1-(trimethylsilyloxy)propan-2-ylcarbamate (**58**):** To a solution of freshly prepared aldehyde **18** (2.16 g, 8.67 mmol), in dry CH_2Cl_2 (30 mL) cooled to -20°C and magnetically stirred under Ar, were subsequently added Lewis acid MgBr_2 (1.61 g, 8.76 mmol) and trimethylsilylcyanide (1.17 mL, 8.76 mmol). After stirring at -20°C for 4 h, the mixture was poured into H_2O (100 mL). The layers were separated and the aqueous layer was extracted 3 times with Et_2O . The combined organic layers were washed with brine (2×100 mL), dried over anhydrous Na_2SO_4 and evaporated in vacuo. The crude mixture was purified using flash chromatography (100% hexane to 100% ethyl acetate in 40 min) furnishing cyanohydrine **58** in moderate yield (0.66 g, 22%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=0.25$ (s, 9H, TMS), 1.41 (s, Boc, 9H), 2.78–3.07 (m, 2H, CH_2Ph), 4.08 (m, 1H, CH), 4.55 (d, $J=3.2$ Hz, 1H, CH(OTMS)), 4.85 (d, $J=8.4$ Hz, 1H, NH), 7.19–7.30 (m, 5H, Ph); MS (ESI) m/z : 371 [$M+\text{Na}$] $^+$.

***tert*-Butyl (2*S*)-4-amino-3-hydroxy-4-(hydroxyimino)-1-phenylbutan-2-ylcarbamate (**59**):** In a 100 mL round-bottomed flask **58** (1.03 g, 2.96 mmol) and hydroxyl amine hydrochloride (0.236 g, 3.40 mmol) were dissolved in MeOH (40 mL) to give a colourless solution. Sodium bicarbonate (0.286 g, 3.40 mmol) was added and the mixture was refluxed for 4 h. Afterwards the mixture was subjected to FCC, (Hex/ EtOAc 1:1) to yield product **59**. (0.28 g, 31%); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.37$ (s, 9H, Boc), 2.95–2.99 (m, 2H, CH_2Ph), 3.98 (m, 1H, CH), 4.27 (s br, 1H, CHOH), 5.07 (s br, 1H, NH), 5.31 (s br, 2H, NH_2), 7.19–7.33 (m, 5H, Ph); MS (ESI) m/z : 310 [$M+\text{H}$] $^+$.

***tert*-Butyl (2*S*)-1-hydroxy-3-phenyl-1-(5-phenyl-1,2,4-oxadiazol-3-yl)propan-2-ylcarbamate (**60**):** In a 100 mL round-bottomed flask benzoic acid (0.079 g, 0.646 mmol), 1-hydroxybenzotriazole (0.096 g, 0.711 mmol) and *N,N'*-dicyclohexylcarbodiimide (0.147 g, 0.711 mmol) were dissolved in DMF (20 mL) to give a yellowish solution. Triethylamine (0.198 mL, 1.422 mmol) was added and the solution was stirred at room temperature for 1 h. Compound **59** (0.2 g, 0.646 mmol) was added and the solution was left stirring for 5 h. A fivefold volume of water (100 mL) was added and the mixture was extracted with ethyl acetate (3×20 mL). The combined organic layers were washed with water (1×50 mL) and brine (1×50 mL). After drying over anhydrous Na_2SO_4 , the solution was filtered and concentrated by rotary evaporation. This crude mixture is then dissolved in pyridine (20 mL) to give a yellow solution. The solution was heated for 15 h at 120°C . Afterwards the excess of pyridine was removed by evaporation and the product was adsorbed on silica and subjected to flash column chromatography (100% hexane to 100% ethyl acetate in 40 min); $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta=1.41$ (s, 9H, Boc), 2.88–3.05 (m, 2H, CH_2Ph), 3.25 (d, $J=6$ Hz, 1H, OH), 4.30 (m, 1H, CH), 4.92 (m, 1H, CHOH), 5.10 (d, $J=8.0$ Hz, 1H, NH), 7.17–7.61 (m, 8H, Ph), 8.09–8.16 (m, 2H, Ph); MS (ESI) m/z : 418 [$M+\text{Na}$] $^+$.

N-[(2*S*)-1-[(2*S*)-1-Hydroxy-3-phenyl-1-(5-phenyl-1,2,4-oxadiazol-3-yl)propan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (**61**): Compound **60** (0.14 g, 0.35 mmol) was used in procedure A, coupled to Mu-Phe-OH and yielded coupled product **61**. (0.15 g, 76%); ¹H-NMR (400 MHz, CDCl₃): δ=2.92-3.18 (m, 4H, 2 × CH₂Ph), 3.25 (m, 4H, CH₂N), 3.56 (m, 4H, CH₂O), 4.55 (m, 1H, CH), 4.71 (m, 1H, CH), 4.86 (s br, 1H, OH), 4.99 (m, 1H, CHOH), 6.82 (d, *J*=8.8 Hz, 1H, NH), 6.89 (d, *J*=8.4 Hz, 1H, NH), 6.97-7.56 (m, 13H, Ph), 7.99-8.04 (m, 2H, Ph); MS (ESI) *m/z*: 418 [*M*+Na]⁺.

N-[(2*S*)-1-[(2*S*)-1-Hydroxy-3-phenyl-1-(5-phenyl-1,2,4-oxadiazol-3-yl)propan-2-ylamino]-1-oxo-3-phenylpropan-2-yl]morpholine-4-carboxamide (**15**): Compound **61** (0.15 g, 0.29 mmol) was used in procedure B and yielded keto-isoxazole **15** as an off-white solid (70 mg, 44%); HPLC (λ=214 nm): *t*_R=24.72 min; ¹H-NMR (400 MHz, CDCl₃): δ=2.97-3.03 (m, 2H, CH₂Ph), 3.10-3.32 (m, 2H, CH₂Ph), 3.27 (m, 4H, CH₂N), 3.62 (m, 4H, CH₂O), 4.60 (m, 1H, CH), 5.08 (d, *J*=7.2 Hz, 1H, NH), 5.59 (m, 1H, CH), 6.70 (d, *J*=6.8 Hz, 1H, NH), 6.99-7.28 (m, 10H, Ph), 7.56-7.68 (m, 3H, Ph), 8.19-8.22 (m, 2H, Ph); ¹³C-NMR (100 MHz, CDCl₃): δ=34.46 (CH₂Ph), 38.56 (CH₂Ph), 43.98 (CH₂NCH₂), 55.39 (CH), 58.01 (CH), 66.39 (CH₂OCH₂), 123.24, 127.06, 127.22, 128.54, 128.67, 128.69, 129.33, 129.53, 133.66, 135.09, 136.85 (Ph), 156.89 (C-3 oxadiazole), 164.91 (C=O urea), 171.56 (C-5 oxadiazole), 177.26 (C=O amide), 188.93 (Het-C=O) ppm; MS (ESI) *m/z*: 576 [*M*+Na]⁺; LC-MS *t*_R=16.9 min, *m/z*: 554 [*M*+H]⁺.

Acknowledgments

We gratefully acknowledge financial support of the Research Fund of the University of Antwerp and the Research Foundation Flanders (FWO Vlaanderen). K. Steert fulfilled a Dehousse mandate financed by a GOA project of the Research Fund of the University of Antwerp. M. Berg has a PhD grant from the Research Foundation Flanders (FWO Vlaanderen). P. Van der Veken and P. Cos are postdoctoral fellows of the Research Foundation Flanders (FWO-Vlaanderen). J. Joossens is Research and Innovation Manager of the Antwerp Drug Discovery Network (ADDN, www.addn.be) funded by the Industrial Research Fund of the University of Antwerp. J.C. Mottram, G.H. Coombs and G.D. Westrop are supported by the Medical Research Council (Grant number G0700127) and an SRDG grant (HR04013) from the Scottish Funding Council. The excellent technical assistance of W. Bollaert is greatly appreciated.

References

- [1] a). World Health Organization (WHO). World Health Report. 2002. <http://www.who.int/whr/2002/enb> Tropical Disease Research (TDR). 2005. <http://www.who.int/tdr/>
- [2]. Sajid M, McKerrow JH. Mol. Biochem. Parasitol. 2002; 120:1–21. [PubMed: 11849701]
- [3]. Lecaille F, Kaleta J, Brömme D. Chem. Rev. 2002; 102:4459–4488. [PubMed: 12475197]
- [4]. Frame MJ, Mottram JC, Coombs GH. Parasitology. 2000; 121(4):367–377. [PubMed: 11072899]
- [5]. Scory S, Caffrey CR, Steirhof YD, Ruppel A, Steverding D. Exp. Parasitol. 1999; 91:327–33. [PubMed: 10092476]
- [6]. Engel JC, Doyle PS, Hsieh I, McKerrow JH. J. Exp. Med. 1998; 188:725–734. [PubMed: 9705954]
- [7]. Barr SC, Warner KL, Kornreic BG, Piscitelli J, Wolfe A, Benet L, McKerrow JH. Antimicrob. Agents. Chemother. 2005; 49:5160–5161. [PubMed: 16304193]
- [8]. Besteiro S, Williams RA, Coombs GH, Mottram JC. Int. J. Parasitol. 2007; 37:1063–1075. [PubMed: 17493624]
- [9]. Brooks DR, Denise H, Westrop GD, Coombs GH, Mottram JC. J. Biol. Chem. 2001; 276:47061–47069. [PubMed: 11592967]
- [10]. Sanderson SJ, Pollock KG, Hilley JD, Meldal M, Hilaire PS, Juliano MA, Juliano L, Mottram JC, Coombs GH. Biochem J. 2000; 347:383–388. [PubMed: 10749667]
- [11]. Mottram JC, Souza AE, Hutchison JE, Carter R, Frame MJ, Coombs GH. Proc Natl Acad Sci U S A. 1996; 93:6008–6013. [PubMed: 8650210]

- [12]. Alexander J, Coombs GH, Mottram JC. *J. Immunol.* 1998; 161:6794–6801. [PubMed: 9862710]
- [13]. Pollock KG, McNeil KS, Mottram JC, Lyons RE, Brewer JM, Scott P, Coombs GH, Alexander J. *Immunol.* 2003; 170:1746–1753.
- [14]. Denise H, McNeil K, Brooks DR, Alexander J, Coombs GH, Mottram JC. *Infect. Immun.* 2003; 71:3190–3195. [PubMed: 12761098]
- [15]. Bryson K, Besteiro S, McGachy HA, Coombs GH, Mottram JC, Alexander J. *Infect. Immun.* 2009; 77:2971–2978. [PubMed: 19433541]
- [16]. Williams RA, Tetley L, Mottram JC, Coombs GH. *Mol. Microbiol.* 2006; 61:655–674. [PubMed: 16803590]
- [17]. Steert K, El-Sayed I, Van der Veken P, Krishtal A, Van Alsenoy C, Westrop GD, Mottram JC, Coombs GH, Augustyns K, Haemers A. *Bioorg. Med. Chem. Lett.* 2007; 17:6563–6566. [PubMed: 17936625]
- [18]. Desai PV, Patny A, Sabnis Y, Tekwani B, Gut J, Rosenthal P, Srivastava A, Avery M. *J. Med. Chem.* 2004; 47:6609–6615. [PubMed: 15588096]
- [19]. Troeberg L, Morty RE, Pike RN, Lonsdale-Eccles JD, Palmer JT, McKerrow JH, Coetzer THT. *Exp. Parasitol.* 1999; 91:349–355. [PubMed: 10092479]
- [20]. Huang L, Brinen LS, Ellman JA. *Bioorg. Med. Chem.* 2003; 11:21–29. [PubMed: 12467703]
- [21]. Choe Y, Brinen LS, Engel JC, Lange M. *Bioorg. Med. Chem.* 2005; 13:2141–2156. [PubMed: 15727867]
- [22]. Palmer JT, Hirschbein BL, Cheung H, McCarter J, Janc JW, Yu ZW, Wesolowski G. *Bioorg. Med. Chem. Lett.* 2006; 16:2909–2914. [PubMed: 16546382]
- [23]. Tavares FX, Deaton DN, Miller LR, Wright LL. *Bioorg. Med. Chem. Lett.* 2005; 15:3891–3895. [PubMed: 15993587]
- [24]. Berg M, Van der Veken P, Joossens J, Muthusamy V, Breugelmans M, Moss CX, Rudolf J, Cos P, Coombs GH, Maes L, Haemers A, Mottram JC, Augustyns K. *Bioorg. Med. Chem. Lett.* 2010; 20:2001–2006. [PubMed: 20167486]
- [25]. Maryanoff BE. *Bioorg. Med. Chem.* 2008; 16:1562–1595. [PubMed: 18053726]
- [26]. Tsutsumi S, Okonogi T, Shibahara S, Ohuchi S, Hatsushiba E, Patchet AA, Cristensen BG. *J. Med. Chem.* 1994; 37:3492–3502. [PubMed: 7932578]
- [27]. Nahm S, Weinreb SM. *Tetrahedron Lett.* 1981; 22:3815–3818.
- [28]. Luzzio FA. *Tetrahedron.* 2001; 57:915–945.
- [29]. Mukaiyama T, Hoshino T. *J. Am. Chem. Soc.* 1960; 82:5339.
- [30]. Ward YD. *J. Med. Chem.* 2002; 45:5471–5482. [PubMed: 12459015]
- [31]. Kornblum N, Larson HO, Blackwood RK, Mooberry DD, Oliveto EP, Graham GE. *Org. React.* 1962; 12:101–156.
- [32]. Wu P, Fokin VV. *Aldrichchimica Acta.* 2007; 40(1):7–17.
- [33]. Lindsay RO, Allen CFH. *Org. Synt.* 1955; Coll. Vol. 3:710–711.
- [34]. Maryanoff BE. *Bioorg. Med. Chem.* 2008; 16:1562–1595. [PubMed: 18053726]
- [35]. Macor JE, Forman JT, Post RJ, Ryan K. *Tetrahedron Lett.* 1997; 38:1673–1676.
- [36]. Sheldrake PW, Matteucci M, McDonald E. *Synlett.* 2006; 3:460–462.
- [37]. Costanzo MJ, Almond HR Jr, Hecker LR, Schott MR, Yabut SC, Zhang H-C, Andrade-Gordon P, Corcoran TW, Giardino EC, Kauffman JA, Lewis JM, de Garavilla L, Haertlein BL, Maryanoff BE. *J. Med. Chem.* 2005; 48:1984–2008. [PubMed: 15771442]
- [38]. Dixon M. *Biochem. J.* 1972; 129:197–202. [PubMed: 4630451]
- [39]. Williams JW, Morrison JF. *Methods Enzymol.* 1979; 63:437–467. [PubMed: 502865]
- [40]. Pippel DJ, Mapes CM, Mani NS. *J. Org. Chem.* 2007; 72:5828–5831. [PubMed: 17585818]
- [41]. Bernardi L, Bonini BF, Dessole G, Fochi M, Varchi G. *J. Org. Chem.* 2003; 68:1418–1425. [PubMed: 12585882]
- [42]. Koo KD, Kim MJ, Kim S, Lee C-S. *Bioorg. Med. Chem. Lett.* 2007; 17:4167–4172. [PubMed: 17544668]
- [43]. Mahmoudzadeh-Niknam H, McKerrow JH. *Exp. Parasitol.* 2004; 106:158–163. [PubMed: 15172223]

- [44]. Selzer PM, Pingel S, Hsieh I, Ugele B, Chan VJ, Engel JC, Bogyo M, Russell DG, Sakanari JA, McKerrow JH. *Proc. Natl. Acad. Sci USA*. 1999; 96:11015–11022. [PubMed: 10500116]
- [45]. Engel JC, Doyle PS, Hsieh I, McKerrow JH. *J. Exp. Med.* 1998; 188:725–734. [PubMed: 9705954]
- [46]. Frame MJ, Mottram JC, Coombs GH. *Parasitol.* 2000; 121:367–377.
- [47]. Pupkis MF, Coombs GH. *Comp. Biochem. Physiol. B.* 1986; 83:629–632. [PubMed: 3956174]
- [48]. McKerrow JH, Rosenthal PJ, Swenerton R, Doyle P. *Curr. Opin. Infect. Dis.* 2008; 21:668–672. [PubMed: 18978536]
- [49]. Cos P, Vlietinck AJ, Berghe DV, Maes L. *J. Ethnopharmacol.* 2006; 106:290–302. [PubMed: 16698208]

Figure 1.
Cysteine protease inhibitor 'warheads' and their molecular basis for activity.

Figure 2.
Generalized structure of target compounds derived from **Mu-K1177**.

Figure 3.

Overview of α -ketoheterocycle-based target cysteine protease inhibitors; *All compounds have $n = 1$ and $X = O$, except compounds **2** and **3**.

Scheme 1.

Synthesis of target compounds **1-5**; (a) *N,O*-dimethylhydroxylamine hydrochloride, TBTU, NEt₃, DMF (b) LiAlH₄, THF, -10°C (c) CH₃NO₂, TBAF, THF, 0°C (d) TMSCl, NEt₃, THF (e) PhNCO, cat. NEt₃, phenylacetylene (**23**, **27**)/3-phenyl-1-propyn (**25**)/trimethylsilylacetylene (**26**), toluene, 90°C, 15h (f) AcOH/H₂O 8:2 (g) TFA, CH₂Cl₂ (1:1) (h) Mu-Phe-OH* (**28-31**)/ *N*-Me-Phe-OH** (**32**), TBTU, NEt₃, DMF (i) (COCl)₂, DMSO, NEt₃, CH₂Cl₂ 0°C. *Mu-Phe-OH: morpholineurea-phenylalanine-OH, ***N*-Me-Phe-OH: *N*-methylpiperazine-phenylalanine-OH

Scheme 2.

Synthesis of target compounds **6** and **7**; (a) NaNO_2 , urea, DMF, -20°C (b) NaNO_2 , HCl, H_2O , Et_2O (c) *N,O*-dimethylhydroxylamine hydrochloride, TBTU, NEt_3 , DMF (d) ethynylmagnesiumbromide, THF, 0°C (e) phenylnitromethane (**34**), PhNCO , NEt_3 , toluene, 50°C (if R = isoxazol-5-yl*), azidobenzene (**36**), CuI , MeOH , 60h, rt (if R = triazol-4-yl**) (f) TFA, CH_2Cl_2 (g) Mu-Phe-OH, EDC, HOBT, NEt_3 , CH_2Cl_2

Scheme 3.

Synthesis of target compounds **8** and **9**; (a) [(azidomethoxy)methyl]benzene, CuI, MeOH, 5h reflux, 12h, rt (b) NaBH₄, MeOH, -20°C, 1h (c) i. TFA, CH₂Cl₂ ii. Mu-Phe-OH, TBTU, NEt₃, CH₂Cl₂ (d) (COCl)₂, DMSO, NEt₃, CH₂Cl₂ 0°C (e) H₂, Pd/C, EtOH, 5 days, rt

Scheme 4.

Synthesis of target compounds **10-12**: (a) $\text{NaN}(\text{CHO})_2$, CH_3CN (b) P_2S_5 , NEt_3 , CHCl_3 , 60°C , 1h (c) $n\text{BuLi}$, thiazole, dry THF, -78°C (if R*), $n\text{BuLi}$, benzo[d]thiazole, dry THF, -78°C (if R**), **46**, LDA, THF, -78°C (if R***), (d) NaBH_4 , MeOH, -10°C (e) i) TFA, CH_2Cl_2 (1:1) ii) Mu-Phe-OH, TBTU, NEt_3 , DMF (f) $(\text{COCl})_2$, DMSO, NEt_3 , CH_2Cl_2 (g) Mu-Phe-OH, EDC, HOBt, NEt_3 , CH_2Cl_2

Scheme 5.

Synthesis of target compounds **13-14**; (a) 5-phenyloxazole (if R*) or benzo[*d*]oxazole (if R**), *i*PrMgCl, dry THF, -15°C (b) i) TFA, CH_2Cl_2 (1:1) ii) Mu-Phe-OH, TBTU, NEt_3 , DMF (c) $(\text{COCl})_2$, DMSO, NEt_3 , CH_2Cl_2

Scheme 6.

Synthesis of target compound **15**; (a) TMSCN, MgBr₂, CH₂Cl₂ (b) hydroxylamine hydrochloride, NaHCO₃, MeOH (c) i) benzoic acid, HOBT, DCC ii) pyridine, 120°C, 14h (d) i) TFA, CH₂Cl₂ ii) Mu-Phe-OH, TBTU, NEt₃, DMF (h) (COCl)₂, DMSO, NEt₃, CH₂Cl₂

Table 1Biochemical activity (IC_{50} and K_i in μM) versus CPB2.8 Δ CTE of *L. mexicana*.

Product	IC_{50} (μM)	K_i (μM)
1a	0.13 ± 0.04	0.004 ± 0.001
1b	0.10 ± 0.04	0.003 ± 0.001
2a	0.19 ± 0.06	0.006 ± 0.002
2b	1.93 ± 0.31	0.062 ± 0.010
3	0.23 ± 0.02	0.0074 ± 0.0005
4	3.66 ± 0.53	0.118 ± 0.017
5a	> 100	> 3.226
5b	> 100	> 3.226
6	1.50 ± 0.22	0.048 ± 0.007
7a	0.44 ± 0.04	0.014 ± 0.001
7b	43.9 ± 18.3	1.416 ± 0.590
8	52.9 ± 9.49	1.706 ± 0.306
9	> 100	> 3.226
10	> 100	> 3.226
11	23.79 ± 1.93	0.767 ± 0.062
12	12.94 ± 1.27	0.417 ± 0.041
13	0.47 ± 0.04	0.015 ± 0.001
14	3.55 ± 0.63	0.115 ± 0.020
15	0.050 ± 0.003	0.0016 ± 0.0001
Mu-K11777	0.027 ± 0.007	-
NMe-K11777	0.054 ± 0.028	-

In calculating K_i values with the Cheng-Prusoff equation ($K_i = IC_{50}/(1 + [S]/K_m)$, the assumption is made that compounds **1-15** are competitive inhibitors (kinetic analysis indicated that compound **15** is a competitive inhibitor). The equation cannot be used to calculate K_i values for the irreversible inhibitors Mu-K11777 and NMe-K11777.

Table 2

IC₅₀-values (μ M) of the target compounds are given for different microorganisms. CC₅₀-values (μ M) of the target compounds are given for MRC-5 human cell line. From left to right: MRC-5 human cell line, *T. cruzi*, *L. infantum*, *T. b. brucei*, *P. falciparum*. Moderate activity is indicated in bold.

Product	MRC-5	<i>T. cruzi</i>	<i>L. inf.</i>	<i>T. brucei</i>	<i>P. falcip.</i>
1a	> 64.00	> 64.00	> 64.00	37.65	> 64.00
1b	> 64.00	> 64.00	> 64.00	32.69	> 64.00
2a	> 64.00	42.63	> 64.00	32.69	> 64.00
2b	> 64.00	46.48	> 64.00	30.75	58.84
3	> 64.00	> 64.00	> 64.00	32.69	> 64.00
4	> 64.00	> 64.00	> 64.00	8.17	> 64.00
5a	> 64.00	> 64.00	> 64.00	6.00	> 64.00
5b	> 64.00	> 64.00	> 64.00	17.00	> 64.00
6	> 64.00	27.97	> 64.00	11.26	12.70
7a	> 64.00	> 64.00	32.46	> 64.00	> 64.00
7b	> 64.00	27.04	50.80	30.87	5.07
8	> 64.00	32.24	> 64.00	> 64.00	17.28
9	> 64.00	> 64.00	> 64.00	> 64.00	34.56
10	> 64.00	31.70	> 64.00	17.33	> 64.00
11	> 64.00	17.48	12.70	8.06	1.89
12	> 64.00	19.76	32.46	8.17	13.00
13	> 64.00	29.13	> 64.00	32.46	> 64.00
14	> 64.00	31.50	> 64.00	32.94	> 64.00
15	> 64.00	27.66	> 64.00	32.46	22.63
Mu-K11777	33.22	< 0.25	> 64.00	< 0.25	27.47
NMe-K11777	33.51	< 0.25	> 64.00	< 0.25	3.65