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# Development of novel dipeptide-like rhodesain inhibitors containing the 3-bromoisoxazoline warhead in a constrained conformation

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ABSTRACT

Novel dipeptide-like rhodesain inhibitors containing the 3-bromoisoxazoline warhead in a constrained conformation were developed; some of them possess $K_i$ values in the micromolar range. We studied the structure–activity relationship of these derivatives and we performed docking studies, which allowed us to find out the key interactions established by the inhibitors with the target enzyme. Biological results indicate that the nature of the P2 and P3 substituents and their binding to the S2/S3 pockets is strictly interdependent.



## 1. Introduction

Neglected tropical diseases are a diverse group of diseases with distinct characteristics that thrive mainly among the poorest populations, affecting more than 1.4 billion people.[1]

In this context, one of the most relevant disease is the Human African Trypanosomiasis (HAT), also known as sleeping sickness. The disease is caused by parasites of *Trypanosoma* genus, transmitted by the bite of a fly of *Glossina* genus. Two are the main subspecies of *Trypanosoma* able to transmit the disease to humans: *T. brucei gambiense*, responsible for the West African or Gambian African sleeping sickness, a chronic form of the disease, and *T. brucei rhodesiense*, which causes the East African sleeping sickness.[2]

At present, there are only few available drugs, that is, suramine and pentamidine, which are active on the first stage of the disease, while the late-stage of HAT has to be treated with either melarsoprol, a highly toxic drug responsible for up to 8% of the deaths, or with a combination of nifurtimox and eflornithine, which is very expensive and difficult to administer.[3]

Taking into consideration all these reasons, there is a considerable need to find new effective drugs.

To address this need, we focused our attention on rhodesain, the cathepsin L-like cysteine protease that has been recognized as a novel promising target for the HAT treatment.[4] Rhodesain (TbCatL) is a clan CA, family C1 (papain-family) cysteine protease that plays essential roles in the life cycle of *T. b. rhodesiense*. It is a single polypeptide chain of 215 amino acids, and it is composed of two domains, left and right, with the catalytic residues $Cys_{25}$/$His_{162}$/$Asn_{182}$ located into a cleft between the two domains.[5]

Rhodesain is localized in the lysosome and it is involved in the degradation of parasite proteins and intracellularly transported host proteins.[6] In addition, it is required by the parasite to cross the blood–brain barrier of the human host causing the second and lethal stage of the disease. Moreover, it is involved in the turnover of the variant surface glycoproteins of the *Trypanosoma* coat, the way to escape the host immune system. Furthermore, it takes part to the degradation of the immunoglobulins, thus preventing a specific response by the host cells.

In this context, our research group has already developed several non-peptidic[7] or peptidomimetic[8,9] parasitic cysteine protease inhibitors.

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In particular, we recently showed the usefulness of the 3-bromoisoxazoline moiety as a novel type of warhead[10] that, coupled to a specific peptidomimetic recognition moiety, gave rise to rhodesain inhibitors with a low micromolar $K_i$ values.[11]

In the present paper, we aimed to demonstrate the wider application of this warhead on dipeptide-like structures, designed in analogy with azadipeptide nitriles,[12] which are well-known rhodesain inhibitors (Fig. 1).

At the P1 position, we inserted a bicyclic scaffold carrying the 3-bromoisoxazoline warhead, whereas at the P2 position we introduced Phe, homoPhe or Leu residues, taking into consideration that, in such a position, rhodesain has a strong preference for hydrophobic residues. Moreover, we investigated the effect of introducing different groups at the P3-site by functionalizing the N-terminal position as a carbamate (compounds **1**–**14**, Fig. 1).

The present paper reports the synthesis, the biological evaluation against rhodesain and *T. b. brucei* and the docking studies of the new compounds **1**–**14**.

## 2. Results and discussion

The methyl esters of amino acids L-Phe, L-homoPhe, and L-Leu (i.e., compounds **15**–**17**) were initially functionalized at the amino group as carbamates **18**–**29**. The ester function of the latter intermediates was then hydrolyzed under alkaline conditions to yield the corresponding carboxylic acids **30**–**41**, which were coupled to racemic amine **42**,[11a] to produce a 1:1 mixture of diastereomers **1a**,**b**–**12a**,**b**; as shown in Scheme 1, the α-amino acid center has *S* configuration. The two pairs of diastereomers could not be separated by conventional chromatographic methods. Therefore, we decided to test the diastereomeric mixtures to gain preliminary information on their biological activity.

The diastereomeric mixtures **1a**,**b**–**12a**,**b** were tested for their inhibitory activity against rhodesain; Cbz-Phe-Arg-7-amino-4-methylcoumarin was used as the fluorogenic substrate.

A preliminary screening with an inhibitor concentration of 100 μM was performed and an equivalent volume of DMSO was used as negative control. Compounds capable of inhibiting the enzymatic activity by more than 40% were progressed to the continuous assays to determine $K_i$ values (Table 1).

The most potent diastereomeric mixture against rhodesain was **5a**,**b**, with a homoPhe residue at P2 site and an Alloc group at the N-terminal, which displayed a $K_i$ value of 2.62 μM. When homoPhe is replaced by Phe (compound **1a**,**b**) or Leu (compound **9a**,**b**), we noticed a substantial decrease in activity ($K_i$ = 16.78 μM and 8.36 μM, respectively). Keeping constant the homoPhe residue at the P2 position, we then explored the effect of the P3 substituent. Surprisingly, by replacing the allyl group with the benzyl, *n*-propyl or *t*-butyl group, the activity was invariably lost (compounds **6a**,**b**–**8a**,**b**). On the contrary, compounds with the Phe at the P2 site can tolerate both the allyl and benzyl group at the P3 site (**1a**,**b** and **2a**,**b**), as well as the derivatives having a Leu at the P2 site, which tolerate both the allyl and *t*-butyl at the P3 site (**9a**,**b** and **12a**,**b**), although for all these compounds the observed activity was lower in comparison to that evaluated on derivative **5a**,**b**.

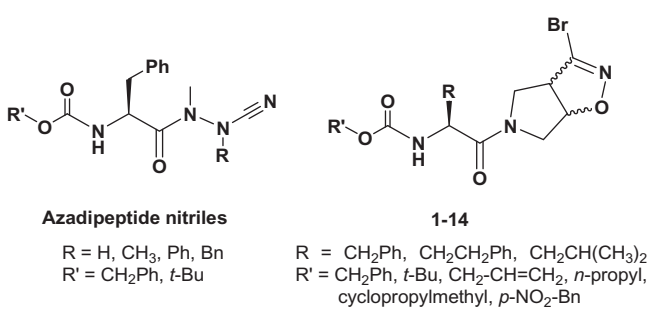



**Figure 1.** Model and target compounds.

In order to evaluate the impact of the stereochemistry of the warhead on the biological activity, we synthesized the single diastereomers (*S*,*R*,*R*)-**1a**, (*S*,*R*,*R*)-**5a**, (*S*,*R*,*R*)-**9a**, and (*S*,*S*,*S*)-**1b**, (*S*,*S*,*S*)-**5b**, and (*S*,*S*,*S*)-**9b**. The synthesis of the single diastereomers was accomplished by coupling carboxylic acids **30**, **34** and **38** with the enantiopure amines (*R*,*R*)-**42** and (*S*,*S*)-**42**, obtained as previously described by us (Scheme 2).[11a] As shown in Table 1, the warhead stereocenters play a marginal role on the biological activity; in all the three cases the diastereomers (*S*,*S*,*S*)-**1b**, (*S*,*S*,*S*)-**5b**, and (*S*,*S*,*S*)-**9b** show $K_i$ values comparable to that of their counterparts (*S*,*R*,*R*)-**1a**, (*S*,*R*,*R*)-**5a** and (*S*,*R*,*R*)-**9a**.

Among the present series of derivatives, the structure–activity relationship of the most interesting compound, that is, compound **5b**, was further investigated by replacing its *N*-allyl group with either the cyclopropylmethyl group (compound **13b**) or the *p*-$NO_2$ benzyl ring (compound **14b**) (Scheme 2).

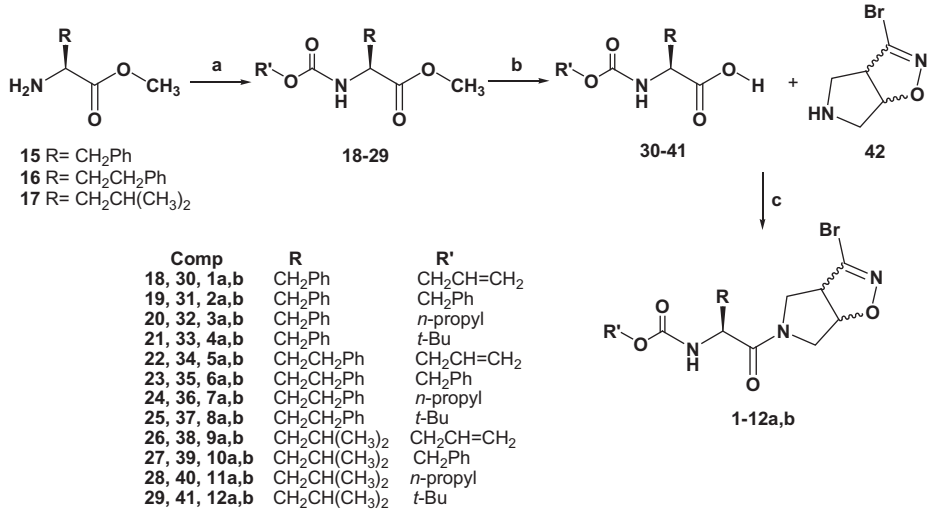
While derivative **13b** turned out to be inactive, the *p*-nitrobenzyl derivative **14b** possesses an affinity for the target enzyme similar to that displayed by reference compound **5b** ($K_i$ = 6.81 μM vs 2.73 μM).

In order to rationalize the structure–activity relationships (SARs) of the derivatives under investigation, we carried out a covalent docking study on compounds **5a** and **5b** with the aid of the Schrodinger package.[13] Although the ligand input files comprised both diastereomers (*S*,*R*,*R*)-**5a** and (*S*,*S*,*S*)-**5b**, the docking program gave a slight preference to the stereoisomer **5b**.

As shown in Figure 2a and b, the homoPhe derivative **5b** fully occupies the S2 pocket, where many hydrophobic contacts with M68, A138, L160 and A208 side chains are detectable. On the other side of the molecule, the Alloc group enters into the S3 pocket with its carbamate function stacking with the well-known planar G65–G66 backbone and the allyl group between the F61 and L67 side chains, with which hydrophobic interactions are detected. The proposed binding mode of **5b** would be in accordance with the SARs herein presented. In fact, docking experiments clearly show that, when the homoPhe moiety fits the S2 pocket, the Alloc moiety is the best tolerated group into the S3 binding site; its small dimension and shape probably play a crucial role. Along these lines, the inactivity of derivatives **6a**,**b**–**8a**,**b** and **13b** can be rationalized through docking studies by considering the bulkiness of both the substituents at P2 and P3, which hampers the simultaneous proper occupation of the two binding pockets. Docking results indicate that the binding of the substituents appended at P2 and P3 to the corresponding S2 and S3 pockets are strictly interdependent. In fact, compounds possessing the Phe residue at the P2 position (i.e., **1a**,**b**–**4a**,**b**) prefer aromatic, flat group at the P3 site (**2a**,**b**) rather than, for instance, the Alloc group (**1a**,**b**), while, as above stated, the opposite holds true for compounds bearing the homoPhe moiety as the P2 group.

Along the same lines, as regards derivatives with Leu at the P2 position (**9a**,**b**–**12a**,**b**), docking studies suggest that each ligand would insert the Leu chain in S2 or in S3 pocket dependently on the P3 substituent type.

As an example, docking of **9a**,**b** suggests a binding mode (see SI) where the Leu chain occupies the S2 pocket, while the Alloc approaches the S3 pocket. Finally, although problems were encountered while docking **14b**, probably due to the rigidity of the target protein in the course of the calculations, it seems likely that **14b** regains activity thanks to the electron withdrawing properties of nitro group which would, on one hand increase the π–π interaction with F61, and on the other hand, it would establish one or more water-bridged interactions with the protein surface.




**Scheme 1.** Reagents and conditions: (a) R′OCOCl, aq $NaHCO_3$/dioxane (7:3), rt, 12 h or $(Boc)_2O$, TEA, $CH_2Cl_2$, 0 °C–rt, 12 h; (b) LiOH, $CH_3OH/H_2O$ (1:1), 0 °C–rt, 8 h; (c) HOBt, EDCI, DIPEA, DMF/$CH_2Cl_2$, 0 °C–rt., 12 h.

**Table 1**
Inhibitory activity of derivatives **1**–**14** against rhodesain

| Compd | Rhodesain $K_i$[a] μM or % of inhibition at 100 μM | Compd | Rhodesain $K_i$[a] μM or % of inhibition at 100 μM |
|---|---|---|---|
| **1a**,**b** | 16.78 ± 0.09 | **7a**,**b** | 15.3% |
| **1a** (*S*,*R*,*R*) | 16.58 ± 0.59 | **8a**,**b** | 5.8% |
| **1b** (*S*,*S*,*S*) | 15.14 ± 0.16 | **9a**,**b** | 8.36 ± 0.63 |
| **2a**,**b** | 8.41 ± 0.09 | **9a** (*S*,*R*,*R*) | 9.05 ± 0.08 |
| **3a**,**b** | 13.1% | **9b** (*S*,*S*,*S*) | 8.52 ± 0.57 |
| **4a**,**b** | 17.73 ± 1.19 | **10a**,**b** | 8.3% |
| **5a**,**b** | 2.62 ± 0.21 | **11a**,**b** | 11.3% |
| **5a** (*S*,*R*,*R*) | 3.35 ± 0.12 | **12a**,**b** | 13.6 ± 0.35 |
| **5b** (*S*,*S*,*S*) | 2.73 ± 0.29 | **13b** (*S*,*S*,*S*) | 15.7% |
| **6a**,**b** | n.i | **14b** (*S*,*S*,*S*) | 6.81 ± 0.23 |

[a] Values are the mean ± SD of $n = 2$ experiments performed in duplicate; n.i.: no inhibition.

Thus, all in all, docking studies would suggest that for each compound, both diastereomers are active because an interchange of the two groups (P2, P3) between the two pockets (S2 and S3) may be possible.

Selectivity assays were also performed, by testing the inhibitors against two papain-family human cysteine proteases, cathepsins B and L. In this case, none of the tested compounds passed the initial screening at 100 μM, thus demonstrating the excellent selectivity of the synthesized inhibitors towards the parasitic proteases.

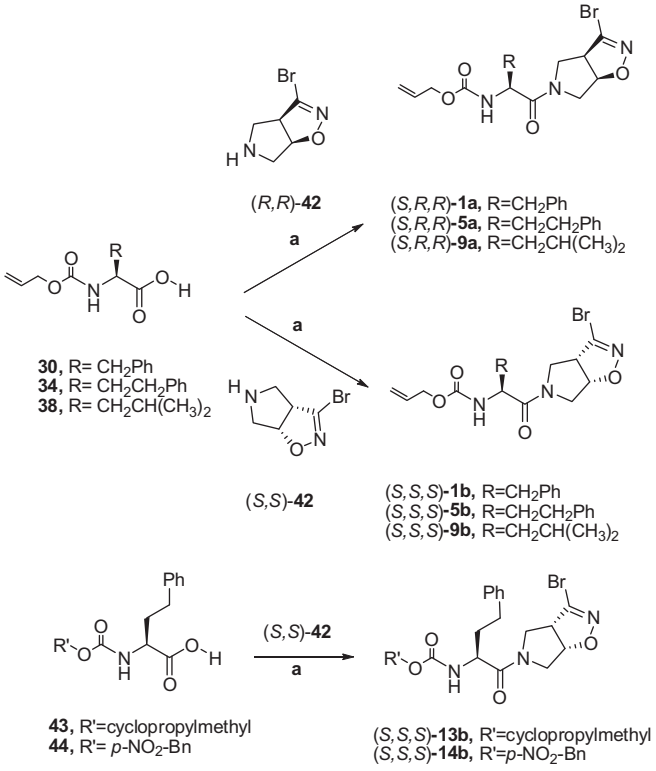
The active inhibitors on rhodesain were tested on *T. b. brucei*, and the ones which showed antitrypanosomal activity were proven to be compound **14b** and the diastereomeric mixture **2a**,**b** ($IC_{50}$ at 48 h = 14.82 ± 0.98 μM and 16.45 ± 1.93 μM, respectively and $IC_{50}$ at 72 h = 14.68 ± 1.98 μM and 18.20 ± 0.06 μM, respectively), probably due to an improved hydrophobic character, which allowed them to better cross the parasite cell membrane. A modest activity was shown also by compound **5a** with an $IC_{50}$ value of 32.04 μM at 48 h.

The cytotoxicity of compounds has also been checked by testing them against mammalian cells by using mouse macrophages (J774.1 cell line) and all of them showed $IC_{50}$ values >100 μM.

## 3. Conclusion

In the present study, we designed novel dipeptide-like rhodesain inhibitors characterized by the presence of the 3-bromoisoxazoline warhead. We varied the nature of the P2 and P3 substituent and found out that their binding to the S2/S3 pocket is strictly interdependent.

Considering that our inhibitors only occupy two (S2/S3) out of the four possible pockets, further substitutions aimed to fit the S1/S1′ pockets might lead to a significant improvement of the inhibitory activity and likely of the antiparasitic activity.




**Scheme 2.** Reagents and conditions: (a) HOBt, EDCI, DIPEA, DMF/$CH_2Cl_2$, 0 °C–rt, 12 h.

## 4. Experimental section

### 4.1. Chemistry

General: All reagents and solvents were obtained from commercial suppliers and were used without further purification.

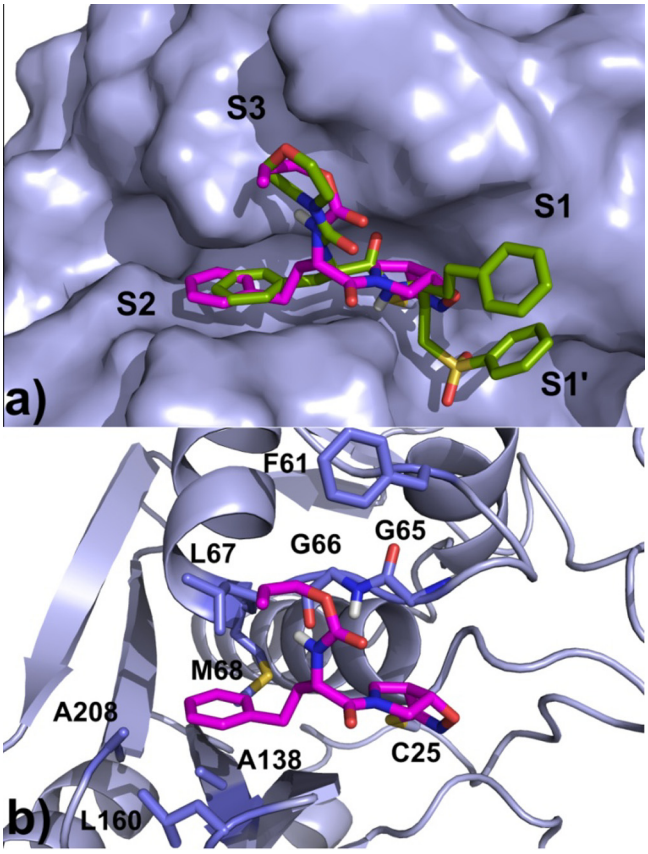



**Figure 2.** (a) Superposition of the binding mode of **5b** (magenta sticks) with the co-crystallized inhibitor K11002 (green sticks). The rhodesain protein is shown as light blue surface. (b) Detailed interactions of **5b** within the rhodesain binding site. The ligand is represented as magenta sticks, while the protein interacting residues as blue sticks.

Elemental analyses (C, H and N) were carried out on a Carlo Erba Elemental Analyzer, Model 1106; the obtained results are within ±0.4% of the theoretical values. Merck silica gel 60 $F_{254}$ plates were used for analytical TLC; flash column chromatography was performed on Merck silica gel (200–400 mesh). $^1$H, and $^{13}$C and NMR spectra were recorded on Bruker Avance 300 MHz NMR spectrometer equipped with a BBI probe and operating at frequencies of 300.13 and 75.47 MHz. We used the residual signal of the deuterated solvent as an internal standard. Splitting patterns are described as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), multiplet (m), or broad singlet (br s). $^1$H and $^{13}$C NMR chemical shifts ($\delta$) are expressed in ppm, and coupling constants ($J$) are given in Hz. MS analyses were performed on a Varian 320-MS triple-quadrupole mass spectrometer equipped with an electron-spray ionization (ESI) source. Polarimetric analyses were carried out on a Perkin–Elmer Polarimeter 341.

### 4.2. General procedure for the synthesis of the *N*-protected carboxylic acids

**Step 1.**

*Procedure a*: To a solution of compounds **15**, **16** or **17** (1 equiv), in a mixture of saturated aqueous solution of $NaHCO_3$/dioxane (7:3, 30 mL) the required chloroformate (1.5 equiv) was added dropwise at 0 °C in a period of 10′. The mixture was then stirred for 12 h, after this time dioxane was evaporated and the reaction mixture was diluted with EtOAc, washed with a 3 N solution of HCl, dried over $Na_2SO_4$ and concentrated in vacuo. The resulting residue was purified by flash column chromatography to remove the excess of chloroformate, using as eluent mixture light petroleum/EtOAc (6:4) to afford pure carbamates.

*Procedure b*: To a solution of compounds **15**, **16** or **17** (1 equiv) in dry $CH_2Cl_2$, TEA (1.5 equiv) and $(Boc)_2O$ (1.2 equiv) were added. The reaction mixture was stirred for 12 h at rt and the solvent was then removed in vacuo. The mixture was diluted with EtOAc, washed with a 3 N solution of HCl, dried over $Na_2SO_4$ and concentrated in vacuo. The resulting residue was purified by flash column chromatography to remove the excess of $(Boc)_2O$, using as eluent a mixture of light petroleum/EtOAc (6:4) to afford pure carbamates.

**Step 2.**

To a solution of the carbamates obtained in the previous step (1 equiv) in a mixture methanol/water (1:1), LiOH (2 equiv) was added at 0 °C. The reaction mixture was then stirred at rt, until disappearance of the starting material (TLC monitoring). The solvent was then concentrated in vacuo and the reaction mixture was treated with 10% citric acid, extracted with EtOAc, dried over $Na_2SO_4$ and concentrated to afford the pure carboxylic acids.

All the spectroscopic data of compounds **30**,[14] **31**,[15] **33**,[16] **34**,[17] **35**,[18] **37**,[19] **38**,[20] **39**,[15] **41**[21] are in agreement to those reported in literature.

#### 4.2.1. (*S*)-3-Phenyl-2-(propoxycarbonylamino)propanoic acid (32)

Overall yield: 89%; $R_f$ = 0.12 ($CH_2Cl_2$/$CH_3OH$, 99:1); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.90 (t, $J$ = 8.0 Hz, 3H), 1.51–1.63 (m, 2H), 3.05–3.12 (m, 1H), 3.15–3.24 (m, 1H), 4.00 (t, $J$ = 7.0 Hz, 2H), 4.64–4.71 (m, 1H), 5.38 (d, $J$ = 7.5 Hz, 1H), 7.16–7.31 (m, 5H), 10.32 (br s, 1H). Anal. calcd for $C_{13}H_{17}NO_4$: C, 62.14; H, 6.82; N, 5.57; found: C, 62.37, H, 6.73; N, 5.66.

#### 4.2.2. (*S*)-4-Phenyl-2-(propoxycarbonylamino)butanoic acid (36)

Overall yield: 87%; $R_f$ = 0.16 ($CH_2Cl_2$/$CH_3OH$, 99:1); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.94 (t, $J$ = 6.8 Hz, 3H), 1.57–1.71 (m, 2H), 1.94–2.11 (m, 1H), 2.15–2.30 (m, 1H), 2.72 (t, $J$ = 7.9 Hz, 2H), 4.04 (t, $J$ = 7.2 Hz, 2H), 4.36–4.48 (m,1H), 5.25 (d, $J$ = 8.3 Hz, 1H), 7.15–7.32 (m, 5H), 10.30 (br s, 1H). Anal. calcd for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28; found: C, 63.18; H, 7.49; N, 5.36.

#### 4.2.3. (*S*)-4-Methyl-2-(propoxycarbonylamino)pentanoic acid (40)

Overall yield: 75%; $R_f$ = 0.11 ($CH_2Cl_2$/$CH_3OH$, 99:1); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.88 (t, $J$ = 7.4 Hz, 3H), 0.92 (d, $J$ = 7.5 Hz, 3H), 0.95 (d, $J$ = 7.3 Hz, 3H), 1.41–1.76 (m, 5H), 4.01(t, $J$ = 7.4 Hz, 2H), 4.31–4.42 (m, 1H), 5.05 (d, $J$ = 6.8 Hz, 1H), 10.31 (br s, 1H). Anal. calcd for $C_{10}H_{19}NO_4$: C, 55.28; H, 8.81; N, 6.45; found: C, 54.97; H, 8.69; N, 6.59.

#### 4.2.4. (*S*)-2-((Cyclopropylmethoxy)carbonylamino)-4-phenylbutanoic acid (43)

Overall yield: 84%; $R_f$ = 0.28 ($CH_2Cl_2$/$CH_3OH$, 99:1); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.22–0.35 (m, 2H), 0.50–0.65 (m, 2H), 0.80–0.91 (m, 1H), 1.97–2.13 (m, 1H), 2.18–2.33 (m, 1H), 2.75 (t, $J$ = 7.9 Hz, 2H), 3.94 (d, $J$ = 7.1 Hz, 2H), 4.38–4.48 (m, 1H), 5.27 (d, $J$ = 7.7 Hz, 1H), 7.13–7.37 (m, 5H). 10.32 (br s, 1H). Anal. calcd for $C_{15}H_{19}NO_4$: C, 64.97; H, 6.91; N, 5.05; found: C, 65.19; H, 7.09; N, 4.89.

#### 4.2.5. (*S*)-2-((4-Nitrobenzyloxy)carbonylamino)-4-phenylbutanoic acid (44)

Overall yield: 82%; $R_f$ = 0.32 ($CH_2Cl_2$/$CH_3OH$, 99:1); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.95–2.15 (m, 1H), 2.15–2.35 (m, 1H), 2.74 (t, $J$ = 8.0, 2H), 4.39–4.51 (m, 1H), 5.22 (s, 2H), 5.56 (d, $J$ = 8.5 Hz, 1H), 7.14–7.35 (m, 5H), 7.51 (d, $J$ = 8.4 Hz, 2H), 8.21 (d, $J$ = 8.4 Hz,

2H) 10.31 (br s, 1H). Anal. calcd for $C_{18}H_{18}N_2O_6$: C, 60.33; H, 5.06; N, 7.82; found: C, 60.04; H, 4.83; N, 8.02.

### 4.3. General procedure for the coupling reaction

To a solution of the *N*-protected carboxylic acid (1 equiv) in DMF/$CH_2Cl_2$ (1:1), HOBt (1.2 equiv), EDCI (1.2 equiv), amine **42**,[11a] (1 equiv) and DIPEA (1.5 equiv) were added at 0 °C. The reaction mixture was stirred for 15 h, diluted with EtOAc, washed with a saturated solution of $NaHCO_3$, dried over $Na_2SO_4$ and concentrated in vacuo. The crude product was then purified by flash column chromatography by using as eluent a mixture of light petroleum/EtOAc (6:4) to give the diastereomeric mixtures **1**–**12a**,**b**.

#### 4.3.1. Allyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (1a) and allyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (1b)

Yield 88%; $R_f$ = 0.40 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 2.86–3.15 (m, 2H), 3.26 (m, 0.5H), 3.27 (d, *J* = 13.0 Hz, 0.5H), 3.36 (d, *J* = 13.0 Hz, 0.5H), 3.52 (m, 0.5H), 3.64–3.83 (m, 1H), 3.83–3.97 (m, 1H), 4.05 (d, *J* = 13.0 Hz, 0.5H), 4.17 (d, *J* = 13.0 Hz, 0.5H), 4.46–4.67 (m, 3H), 5.21 (m, 2H), 5.29 (m, 1H), 5.50 (d, *J* = 8.8 Hz, 1H), 5.79–5.98 (m, 1H), 7.09–7.36 (m, 5H); MS: *m*/*z* 422.0 $[M+H]^+$; Anal. calcd for $C_{18}H_{20}BrN_3O_4$: C, 51.20; H, 4.77; N, 9.95; found: C, 51.48; H, 4.53; N, 10.12.

#### 4.3.2. Benzyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (2a) and benzyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (2b)

Yield 92%; $R_f$ = 0.42 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 2.89–3.18 (m, 2H), 3.23–3.34 (m, 0.5H), 3.30 (d, *J* = 12.1 Hz, 0.5H), 3.38 (d, *J* = 12.1 Hz, 0.5H), 3.46–3.60 (m, 0.5H), 3.66–3.84 (m, 1H), 3.86–3.99 (m, 1H), 4.07 (d, *J* = 12.1 Hz, 0.5H), 4.19 (d, *J* = 12.1 Hz, 0.5H), 4.57–4.73 (m, 1H), 5.05–5.12 (m, 0.5H),5.14 (s, 2H), 5.18–5.29 (m, 0.5H), 5.54 (d, *J* = 7.4 Hz, 1H), 7.10–7.46 (m, 10H); MS: *m*/*z* 472.1 $[M+H]^+$; Anal. calcd for $C_{22}H_{22}BrN_3O_4$: C, 55.94; H, 4.69; N, 8.90; found: C, 55.72; H, 5.03; N, 8.77.

#### 4.3.3. *n*-Propyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (3a) and *n*-propyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (3b)

Yield 84%; $R_f$ = 0.39 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.93 (t, *J* = 8.0 Hz, 3H), 1.55–1.68 (m, 2H), 2.88–3.02 (m, 1H), 3.02–3.15 (m, 1H), 3.37–3.46 (m, 0.5H), 3.46–3.56 (m, 0.5H), 3.61–3.84 (m, 2H), 3.97 (t, *J* = 7.0, 2H), 3.99–4.09 (m, 1H), 4.10 (d, *J* = 13.8 Hz, 0.5H), 4.17 (d, *J* = 13.8 Hz, 0.5H), 4.52–4.68 (m, 1H), 5.17–5.26 (m, 1H), 5.42 (d, *J* = 7.4 Hz, 1H), 7.11–7.33 (m, 5H); MS: *m*/*z* 424.1 $[M+H]^+$; Anal. calcd for $C_{18}H_{22}BrN_3O_4$: C, 50.95; H, 5.23; N, 9.90; found: C, 51.10; H, 5.46; N, 9.63.

#### 4.3.4. *tert*-Butyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (4a) and *tert*-butyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate (4b)

Yield 90%; $R_f$ = 0.46 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$):$\delta$ = 1.44 (s, 9H), 2.86–3.17 (m, 2H), 3.38–3.49 (m, 0.5H), 3.54 (m, 0.5H), 3.62–4.00 (m, 3H), 4.06 (d, *J* = 15.3 Hz, 0.5H), 4.19 (d, *J* = 15.3 Hz, 0.5H), 4.50–4.69 (m, 1H), 5.19–5.31 (m, 1H), 5.37 (br s, 1H), 7.12–7.41 (m, 5H); MS: *m*/*z* 438.1 $[M+H]^+$; Anal. calcd for $C_{19}H_{24}BrN_3O_4$: C, 52.06; H, 5.52; N, 9.59; found: C, 51.97; H, 5.23; N, 9.86.

#### 4.3.5. Allyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (5a) and allyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (5b)

Yield 85%; $R_f$ = 0.46 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.87–2.13 (m, 2H), 2.62–2.81 (m, 2H), 3.42 (m, 0.5H), 3.60–3.69 (m, 2H), 3.69–3.78 (m, 0.5H), 3.90–4.03 (m, 1H), 4.16 (d, *J* = 18.8 Hz, 0.5H), 4.30 (d, *J* = 18.8 Hz, 0.5H), 4.34–4.50 (m, 1H), 4.54–4.69 (m, 2H), 5.20–5.40 (m, 3H), 5.50 (br s, 1H), 5.86–6.03 (m, 1H), 7.18–7.38 (m, 5H). MS: *m*/*z* 436.1 $[M+H]^+$; Anal. calcd for $C_{19}H_{22}BrN_3O_4$: C, 52.30; H, 5.08; N, 9.63; found: C, 52.51; H, 5.39; N, 9.42.

#### 4.3.6. Benzyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (6a) and benzyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (6b)

Yield 87%; $R_f$ = 0.42 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.88–2.13 (m, 2H), 2.62–2.87 (m, 2H), 3.30–3.42 (m, 0.5H), 3.42–3.53 (m, 0.5H), 3.59–3.74 (m, 2H), 3.86–4.04 (m, 1H), 4.10–4.26 (m, 1H), 4.36–4.60 (m, 1H), 5.14 (s, 2H), 5.22–5.33 (m, 1H), 5.52 (br s, 1H), 7.16–7.46 (m, 10H); MS: *m*/*z* 486.1 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}BrN_3O_4$: C, 56.80; H, 4.97; N, 8.64; found: C, 56.94; H, 4.86; N, 8.39.

#### 4.3.7. *n*-Propyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (7a) and *n*-propyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (7b)

Yield 88%; $R_f$ = 0.45 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.96 (t, *J* = 8.0 Hz, 3H), 1.59–1.71 (m, 2H), 1.89–2.07 (m, 2H), 2.65–2.77 (m, 2H), 3.31–3.42 (m, 0.5H), 3.42–3.52 (m, 0.5H), 3.59–3.76 (m, 2H), 3.90–3.99 (m, 1H), 4.03 (t, *J* = 7.5 Hz, 2H), 4.16–4.30 (m, 1H), 4.33–4.49 (m, 1H), 5.22–5.34 (m, 1H), 5.41 (br s, 1H), 7.17–7.39 (m, 5H); MS: *m*/*z* 438.1 $[M+H]^+$; Anal. calcd for $C_{19}H_{24}BrN_3O_4$: C, 52.06; H, 5.52; N, 9.59; found: C, 52.15; H, 5.19; N, 9.85.

#### 4.3.8. *tert*-Butyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (8a) and *tert*-Butyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate (8b)

Yield 96%; $R_f$ = 0.48 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.46 (s, 9H), 1.83–2.11 (m, 2H), 2.61–2.84 (m, 2H), 3.32–3.42 (m, 0.5H), 3.42–3.54 (m, 0.5H), 3.56–3.79 (m, 2H), 3.88–4.06 (m, 1H), 4.11–4.26 (m, 1H), 4.26–4.53 (m, 1H), 5.18–5.33 (m, 1H), 5.37 (br s, 1H), 7.17–7.37 (m, 5H); MS: *m*/*z* 452.1 $[M+H]^+$; Anal. calcd for $C_{20}H_{26}BrN_3O_4$: C, 53.10; H 5.79; N, 9.29; found: C, 53.28; H, 9.42; N, 9.02.

#### 4.3.9. Allyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (9a) and allyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (9b)

Yield 91%; $R_f$ = 0.48 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.97 (d, *J* = 6.7 Hz, 3H), 1.01 (d, *J* = 5.5, 3H), 1.38–1.47 (m, 1H), 1.47–1.54 (m, 1H), 1.66–1.81 (m, 1H), 3.46–3.57 (m, 0.5H), 3.57–3.69 (m, 0.5H), 3.87–4.14 (m, 3H), 4.22 (d, *J* = 13.7 Hz, 1H), 4.41–4.53 (m, 1H), 4.52–4.63 (m, 2H), 5.27

(m, 2H), 5.32–5.45 (m, 2H), 5.83–6.01 (m, 1H); MS: *m*/*z* 388.1 [M+H]$^+$; Anal. calcd for $C_{15}H_{22}BrN_3O_4$: C, 46.40; H, 5.71; N, 10.82; found: C, 46.73; H, 5.62; N, 10.69.

#### 4.3.10. Benzyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (10a) and benzyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (10b)

Yield 83%; $R_f$ = 0.56 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.96 (d, *J* = 6.5 Hz, 3H), 1.01 (d, *J* = 6.7 Hz, 3H), 1.46–1.59 (m, 2H), 1.66–1.80 (m, 1H), 3.42–3.54 (m, 0.5H), 3.55–3.68 (m, 0.5H), 3.71–3.84 (m, 1H), 3.86–4.13 (m, 2H), 4.21 (d, *J* = 13.8 Hz, 1H), 4.42–4.63 (m, 1H), 5.11 (s, 2H), 5.27–5.52 (m, 2H), 7.30–7.47 (m, 5H); MS: *m*/*z* 438.1 [M+H]$^+$; Anal. calcd for $C_{19}H_{24}BrN_3O_4$: C, 52.06; H, 5.52; N, 9.59; found: C, 51.72; H, 5.39; N, 9.76.

#### 4.3.11. *n*-Propyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (11a) and *n*-propyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (11b)

Yield 86%; $R_f$ = 0.50 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.91 (d, *J* = 7.5 Hz, 3H), 0.96 (d, *J* = 7.3 Hz, 3H), 1.02 (t, *J* = 7.4 Hz, 3H), 1.48–1.80 (m, 5H), 3.47–3.84 (m, 2H), 3.87–4.15 (m, 4H), 4.21 (d, *J* = 13.4 Hz, 1H), 4.39–4.61 (m, 1H), 5.19–5.44 (m, 2H); MS: *m*/*z* 390.1 [M+H]$^+$; Anal. calcd for $C_{15}H_{24}BrN_3O_4$: C, 46.16; H, 6.20; N, 10.77; found: C, 46.02; H, 6.43; N, 10.62.

#### 4.3.12. *tert*-Butyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate (12a) and *tert*-butyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate(12b)

Yield 93%; $R_f$ = 0.58 (EtOAc/light petroleum, 6:4); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.96 (d, *J* = 6.7 Hz, 3H), 1.00 (d, *J* = 7.4 Hz, 3H), 1.45 (s, 9H), 1.54–1.67 (m, 2H), 1.66–1.79 (m, 1H), 3.46–3.59 (m, 0.5H), 3.59–3.69 (m, 0.5H), 3.91–4.12 (m, 3H), 4.21 (d, *J* = 14.9 Hz, 1H), 4.36–4.55 (m, 1H), 5.06–5.20 (m, 1H), 5.20 (d, *J* = 7.9 Hz, 1H); MS: *m*/*z* 404.1 [M+H]$^+$; Anal. calcd for $C_{16}H_{26}BrN_3O_4$: C, 47.53; H, 6.48; N, 10.39; found: C, 47.21; H, 6.32; N, 10.58.

### 4.4. General procedure for the synthesis of compounds 1a, 1b, 5a, 5b, 9a, 9b, 13b and 14b

According to the procedure described for the synthesis of **1**–**12a**,**b**, the carboxylic acid **30**, **34**, **38**, **43** or **44** was reacted with the enantiopure amines (*R*,*R*)-**42**[11a] or (*S*,*S*)-**42**[11a] to give the desired compounds.

#### 4.4.1. Allyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate [(*S*,*R*,*R*)-1a]

Yield 89%; $R_f$ = 0.40 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = −51.2 (*c* = 0.40, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 2.86–3.19 (m, 2H), 3.27 (m, 1H), 3.38 (d, *J* = 11.8 Hz, 1H), 3.67–3.85 (m, 1H), 3.88–4.03 (m, 1H), 4.19 (d, *J* = 11.8 Hz, 1H), 4.51–4.69 (m, 3H), 5.22 (m, 2H), 5.30 (m, 1H), 5.58 (d, *J* = 8.8 Hz, 1H), 5.81–6.00 (m, 1H), 7.13–7.38 (m, 5H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 40.40, 48.73, 53.38, 55.87, 57.54, 66.82, 85.43, 118.42, 128.80, 129.05, 129.47, 134.02, 136.05, 137.24, 155.80, 171.82; MS: *m*/*z* 422.2 [M+H]$^+$; Anal. calcd for $C_{18}H_{20}BrN_3O_4$: C, 51.20; H, 4.77; N, 9.95; found: C, 51.39; H, 4.62; N, 10.24.

#### 4.4.2. Allyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-3-phenylpropan-2-ylcarbamate [(*S*,*S*,*S*)-1b]

Yield 92%; $R_f$ = 0.40 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = +42.7 (*c* = 0.18, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 2.83–3.16 (m, 2H), 3.34 (d, *J* = 12.30 Hz, 1H), 3.54 (m, 1H), 3.73–3.96 (m, 2H), 4.07 (d, *J* = 12.30 Hz, 1H), 4.51–4.72 (m, 3H), 5.19–5.37 (m, 3H), 5.51 (d, *J* = 7.6 Hz, 1H), 5.82–6.00 (m, 1H), 7.13–7.36 (m, 5H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 40.42, 48.71, 53.41, 55.82, 57.50, 66.79, 85.38, 118.40, 128.78, 129.10, 129.51, 134.08, 136.08, 137.20, 155.78, 171.77; MS: *m*/*z* 422.2 [M+H]$^+$; Anal. calcd for $C_{18}H_{20}BrN_3O_4$: C, 51.20; H, 4.77; N, 9.95; found: C, 51.33; H, 4.59; N, 10.07.

#### 4.4.3. Allyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate [(*S*,*R*,*R*)-5a]

Yield 81%; $R_f$ = 0.46 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = −70.5 (*c* = 0.39, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.87–2.06 (m, 2H), 2.60–2.82 (m, 2H), 3.42 (m, 1H), 3.57–3.77 (m, 1H), 3.68 (d, *J* = 13.4 Hz, 1H), 3.88–4.05 (m, 1H), 4.13 (d, *J* = 13.4 Hz, 1H), 4.39–4.55 (m, 1H), 4.54–4.66 (m, 2H), 5.20–5.32 (m, 1H), 5.28 (m, 2H), 5.62 (d, *J* = 8.1 Hz, 1H), 5.85–6.01 (m, 1H), 7.17–7.38 (m, 5H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 29.50, 31.74, 47.91, 51.40, 53.09, 55.63, 66.11, 84.43, 117.22, 125.89, 128.68, 129.35, 133.11, 139.21, 141.28, 156.58, 171.78; MS: *m*/*z* 436.1 [M+H]$^+$; Anal. calcd for $C_{19}H_{22}BrN_3O_4$: C, 52.30; H, 5.08; N, 9.63; found: C, 52.56; H, 5.42; N, 9.55.

#### 4.4.4. Allyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate [(*S*,*S*,*S*)-5b]

Yield 85%; $R_f$ = 0.46 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = +60.4 (*c* = 0.22, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.88–2.13 (m, 2H), 2.63–2.78 (m, 2H), 3.46 (m, 1H), 3.60–3.72 (m, 2H), 3.94 (m, 1H), 4.21 (d, *J* = 13.0 Hz, 1H), 4.33–4.45 (m, 1H), 4.55–4.63 (m, 2H), 5.20–5.39 (m, 3H), 5.60 (d, *J* = 7.7 Hz, 1H), 5.85–6.01 (m, 1H), 7.15–7.38 (m, 5H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 29.57, 31.77, 47.93, 51.45, 53.15, 55.69, 66.08, 84.48, 117.28, 125.85, 128.62, 129.37, 133.09, 139.24, 141.26, 156.60, 171.80; MS: *m*/*z* 436.1 [M+H]$^+$;Anal. calcd for $C_{19}H_{22}BrN_3O_4$: C, 52.30; H, 5.08; N, 9.63; found: C, 52.44; H, 5.33; N, 9.47.

#### 4.4.5. Allyl (*S*)-1-((3a*R*,6a*R*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate [(*S*,*R*,*R*)-9a]

Yield 93%; $R_f$ = 0.48 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = −79.3 (*c* = 0.75, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.94 (d, *J* = 6.5 Hz, 3H), 1.01 (d, *J* = 6.7 Hz, 3H), 1.31–1.45 (m, 1H), 1.44–1.62 (m, 1H), 1.64–1.81 (m, 1H), 3.58 (m, 1H), 3.67–3.82 (m, 1H), 3.96–4.11 (m, 1H), 4.00 (d, *J* = 14.1 Hz, 1H), 4.17 (d, *J* = 14.1 Hz, 1H), 4.41–4.50 (m, 1H), 4.50–4.62 (m, 2H), 5.24 (m, 2H), 5.24–5.42 (m, 1H), 5.49 (d, *J* = 8.1 Hz, 1H), 5.82–5.98 (m, 1H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 21.75, 23.82, 26.50, 44.22, 47.91, 51.63, 53.73, 57.96, 65.93, 84.15, 119.09, 134.91, 139.68, 157.45, 172.23; MS: *m*/*z* 388.3 [M+H]$^+$; Anal. calcd for $C_{15}H_{22}BrN_3O_4$: C, 46.40; H, 5.71; N, 10.82; found: C, 46.65; H, 5.43; N, 10.67.

#### 4.4.6. Allyl (*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-4-methyl-1-oxopentan-2-ylcarbamate [(*S*,*S*,*S*)-9b]

Yield 87%; $R_f$ = 0.48 (EtOAc/light petroleum, 6:4): $[\alpha]_D^{20}$ = + 62.2 (*c* = 0.36, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.94 (d, *J* = 6.5 Hz, 3H), 0.99 (d, *J* = 6.1 Hz, 3H), 1.38–1.62 (m, 2H), 1.63–1.77 (m, 1H), 3.51 (m, 1H), 3.90–4.03 (m, 1H), 3.99 (d, *J* = 12.2 Hz, 1H), 4.15–4.28 (m, 1H), 4.19 (d, *J* = 12.2 Hz, 1H), 4.39–4.50 (m, 1H), 4.51–4.63

(m, 2H), 5.25 (m, 2H), 5.32–5.51 (m, 2H), 5.82–5.99 (m, 1H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 21.70, 23.79, 26.52, 44.19, 47.89, 51.61, 53.75, 57.93, 65.95, 84.09, 119.11, 134.92, 139.63, 157.40, 172.12; MS: *m*/*z* 388.3 $[M+H]^+$; Anal. calcd for $C_{15}H_{22}BrN_3O_4$: C, 46.40; H, 5.71; N, 10.82; found: C, 46.23; H, 5.88; N, 10.59.

#### 4.4.7. Cyclopropylmethyl-(*S*)-1-((3aS,6aS)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate [(*S*,*S*,*S*)-13b]

Yield 78%; $R_f$ = 0.38 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = + 65.0 (*c* = 0.56, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 0.24–0.36 (m, 2H), 0.53–0.64 (m, 2H), 0.83–0.97 (m, 1H), 1.86–2.12 (m, 2H), 2.62–2.80 (m, 2H), 3.47 (m, 1H), 3.62–3.71 (m, 2H), 3.86–4.00 (m, 3H), 4.22 (d, *J* = 12.7 Hz, 1H), 4.33–4.44 (m, 1H), 5.24–5.31 (m, 1H), 5.45 (d, *J* = 8.4 Hz, 1H), 7.18–7.38 (m, 5H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 4.10, 10.83, 32.70, 35.26, 48.73, 51.79, 54.33, 57.06, 71.51, 85.66, 127.05, 129.33, 129.83, 139.61, 141.87, 157.45, 173.95; MS: *m*/*z* 450.1 $[M+H]^+$; Anal. calcd for $C_{20}H_{24}BrN_3O_4$: C, 53.34; H, 5.37; N, 9.33; found: C, 53.62; H, 5.19; N, 9.19.

#### 4.4.8. 4-Nitrobenzyl-(*S*)-1-((3a*S*,6a*S*)-3-bromo-3a,4,6,6a-tetrahydropyrrolo[3,4-*d*]isoxazol-5-yl)-1-oxo-4-phenylbutan-2-ylcarbamate [(*S*,*S*,*S*)-14b]

Yield 92%; $R_f$ = 0.23 (EtOAc/light petroleum, 6:4); $[\alpha]_D^{20}$ = + 55.5 (*c* = 1.32, $CH_2Cl_2$); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.89–2.12 (m, 2H), 2.61–2.80 (m, 2H), 3.45 (m, 1H), 3.60–3.69 (m, 2H), 3.96 (m, 1H), 4.23 (d, *J* = 12.8 Hz, 1H), 4.35–4.45 (m, 1H), 5.22 (s, 2H), 5.25–5.31 (m, 1H), 5.65 (d, *J* = 7.6 Hz, 1H), 7.19–7.37 (m, 5H), 7.53 (d, *J* = 8.3 Hz, 2H), 8.25 (d, *J* = 8.3 Hz, 2H); $^{13}$C (75 MHz, $CDCl_3$): $\delta$ = 30.43, 33.61, 48.43, 51.91, 54.03, 56.38, 65.61, 87.86, 123.79, 126.97, 128.33, 129.61, 130.14, 139.45, 140.50, 142.85, 145.80, 157.68, 171.98; MS: *m*/*z* 531.1 $[M+H]^+$; Anal. calcd for $C_{23}H_{23}BrN_4O_6$: C, 51.99; H, 4.36; N, 10.54; found: C, 52.24; H, 4.47; N, 10.29.

### 4.5. Biology

#### 4.5.1. Enzyme assays

The preliminary screening was performed with 100 μM inhibitor concentration using an equivalent amount of DMSO as negative control. The enzyme was recombinantly expressed as previously described.[6] Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC, 10 μM) was determined continuously over a period of 10 min. Compounds showing at least 40% inhibition at 100 μM were subjected to detailed assays. These were performed in a 50 mM sodium acetate buffer, pH 5.5 containing 10 mM DTT with Cbz-Phe-Arg-AMC (10 μM) as substrate.[22] The $K_m$ value used to calculate $K_i$ values from $IC_{50}$ values were determined to 0.9 μM (rhodesain).[23]

Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed twice in 96-well-plates in a total volume of 200 μL. Fluorescence of the product AMC of the substrate hydrolyses was measured using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) at room temperature with a 380 nm excitation filter and a 460 nm emission filter. The dissociation constants $K_i$ of the non covalent complex E·I were obtained from progress curves (10 min) at various concentrations of inhibitor by fitting the progress curves to the 4-parameter $IC_{50}$ equation:

$$y = \frac{y_{max} - y_{min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^s} + y_{min}$$

with *y* [dF/min] as the substrate hydrolysis rate, $y_{max}$ as the maximum value of the dose–response curve, that is, observed at very low inhibitor concentrations, $y_{min}$ as the minimum value, that is, obtained at high inhibitor concentrations, and *s* denotes the Hill coefficient,[24] and correction to zero substrate concentration from $K_i = IC_{50}/(1 + [S]\ K_m^{-1})$.

Assays with cathepsins B and L were performed as described previously.[25] Cbz-Phe-Arg-AMC was used as substrate (80 μM for cathepsin B, 5 μM for cathepsin L). The $K_m$ values used to calculate $K_i$ values from $IC_{50}$ values were 150 μM (cathepsin B) and 6.5 μM (cathepsin L).

#### 4.5.2. Drug screening on *T. b. brucei* cultures

Trypomastigote forms of *T. b. brucei* laboratory strain TC 221 were cultured in Baltz medium according to standard conditions.[26] Trypanocidal activity was determined using the Alamar Blue assay[27,28] in a 96-well plate format and a MR 700 Microplate ELISA Reader. Trypanosomes were added to culture medium containing various concentrations of test compound and 1% solvent to give a cell concentration of $10^4$ cells $mL^{-1}$ in a final volume of 200 μL. Positive and negative controls comprised wells containing medium, 1% solvent and trypanosomes, and wells with test compounds but without trypanosomes, respectively. After 24 h, 20 μL of Alamar Blue were added to each well and the plates were incubated again for a further 24 h or 48 h. Absorbance were then measured at 550 nm with a reference wavelength of 630 nm. $IC_{50}$ values were calculated by linear interpolation as described.[29] Experiments were repeated twice.

#### 4.5.3. Cytotoxicity on J774.1 Macrophages[30]

The macrophage cell line J774.1 was maintained in complete Click RPMI medium. For the experimental procedures, cells were detached from the flasks with a rubber police, washed twice with PBS, and suspended at $2 \times 10^6$ cells per mL in complete Click RPMI medium. J774.1 macrophages were plated in complete RPMI medium (200 μL) without phenol red in 96-well plates in the absence or presence of various concentrations of the compounds and incubated for 24 h at 37 °C, 5% $CO_2$, 95% humidity. Following the addition of AlamarBlue (20 μL), the plates were further incubated at similar conditions. The plates were then read 24 and 48 h later. Control experiments to examine the effect of cell density, incubation time, and DMSO concentration were performed. Absorbance in the absence of compounds was set as 100% of growth control.

### 4.6. Molecular modeling

Among the three Rhodesain X-rays structures in complex with covalent inhibitors, the 2P86 PDB structure has been chosen for our docking studies due to its resolution and the similarity between its co-crystallized inhibitor and our molecules. The 3D structures of the compounds were generated with the Maestro Build Panel and prepared with the Ligprep module, which automatically generates the protonation state at pH7 ± 1.[31] The target rhodesain structure (PDB code: 2P86) was prepared through the Protein Preparation Wizard of the graphical user interface Maestro 9.2.112.[32] and the OPLS-2001 force field. Water molecules were removed and hydrogen atoms were added. Covalent docking have been carried out using the Schrodinger protein modeling and refinement package Prime.[13] Here OPLS2005 force field with implicit solvation and a distance-dependent dielectric of 2R was employed. The exploration of the conformational space of the ligand and Cys25 is done using a procedure similar to that employed in loop refinement in Prime.[32]

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.09.029.

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