ChemPubSoc Europe

DOI: 10.1002/cmdc.201500204

CHEMMEDCHEM Communications

Dipeptidyl Enoates As Potent Rhodesain Inhibitors That Display a Dual Mode of Action

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Dipeptidyl enoates were prepared through a high-yielding two-step synthetic route. They have a dipeptidic structure with a 4-oxoenoate moiety as a warhead with multiple reactive sites. Dipeptidyl enoates were screened against rhodesain and human cathepsins B and L, and were found to be potent and selective inhibitors of rhodesain. Among them (*S*,*E*)-ethyl 5-((*S*)-2-{[(benzyloxy)carbonyl]amino}-3-phenylpropanamido)-7-methyl-4-oxooct-2-enoate (**6**) was the most potent, with an IC_{50} value of 16.4 nM and $k_{inact}/K_i = 1.6 \times 10^6\ M^{-1}\,s^{-1}$ against rhodesain. These dipeptidyl enoates display a reversible mode of inhibition at very low concentrations and an irreversible mode at higher concentrations. Inhibition kinetics data, supported by docking studies, suggest a dual mode of action via attack of cysteine thiolate at two reactive positions.

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a parasitic disease caused by two subspecies of protozoa of the genus *Trypanosoma*: *T. brucei gambiense* and *T. brucei rhodesiense*.[1] *T. b. gambiense* is responsible for the chronic form of HAT in western and central Africa, whereas *T. b. rhodesiense* causes the acute form of the disease in eastern and southern Africa.[2] Currently more than 60 million people living in 36 sub-Saharan countries are at risk of contracting the disease.[3] The estimated number of actual cases is 30 000.[4] Current treatments rely on medications that are toxic, painful, expensive, and which are becoming ineffective due to parasite resistance. Thus, owing to limited healthcare and poor treatment options, infection typically results in death. Development of effective and safe treatments is desperately needed for this devastating disease.[5] The cathepsin-L-like enzyme rhodesain has been identified as a potential target in the search for new drugs against HAT.[6]

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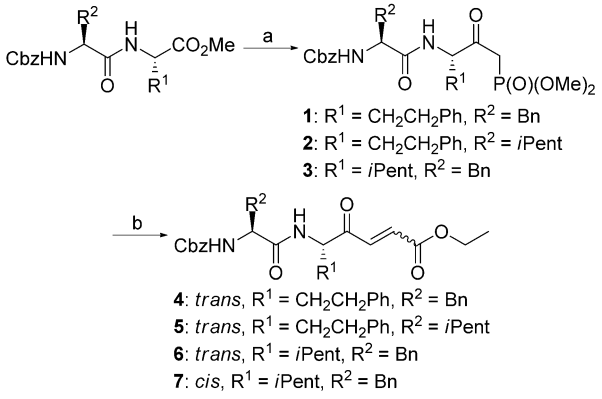
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201500204.

Michael acceptors have been used before as rhodesain inhibitors.[7] For example, dipeptidyl vinylsulfones such as K11777 (Figure 1) are potent irreversible inhibitors of cysteine proteases and rhodesain in particular.[8] We envisioned inhibitors with a Michael-acceptor warhead bearing a carbonyl group between the conjugated double bond and the original α-carbon atom of the P1 residue. The carbonyl group would be placed at the same position as the substrate amide carbonyl group (Figure 1).

A short and efficient synthetic route was developed to prepare the desired inhibitors. The route starts from the corresponding doubly protected dipeptide, which, upon reaction with lithium phosphonate methylide, affords the corresponding phosphonates **1**–**3**.[9] A Horner–Wadsworth–Emmons-type reaction between the phosphorous ylide derived from the phosphonate and ethyl glyoxal furnished the desired inhibitors **4**–**7** (Scheme 1); *trans*-alkenes were obtained in all cases. During the preparation of *trans*-alkene **6**, *cis*-isomer **7** was also isolated.

The dipeptidyl enoates **4**–**7** were screened against rhodesain (Table 1). IC_{50} determinations indicated that compounds **4**, **6**, and **7** are potent inhibitors of rhodesain, with **6** being the most potent. Compound **5**, with an L-leucine residue at the P2 site, was less active. Kinetic analyses were also performed on the compounds. Compound **6** displayed a second-order rate constant higher than that of K11777. Inhibitor **5** is one order of magnitude less active against rhodesain than **4** in terms of both IC_{50} and k_{inact}/K_i, denoting a preference for an L-phenylalanine residue rather than L-leucine at the P2 site. Inhibitor **6**



Scheme 1. Synthesis of inhibitors. *Reagents and conditions*: a) $CH_3C(O)CH_2P(O)(OCH_3)_2$, *n*BuLi, THF, −78 °C, 92–98%; b) ethyl glyoxal, EtOH, K_2CO_3, 76–84%.

Table 1. Inhibition parameters for compounds **4**–**9**.[a]

Inhibitor	Rhodesain		Cathepsin B		Cathepsin L	
	IC_{50} [nM]	k_{inact}/K_i [$M^{-1}s^{-1}$]	IC_{50} [nM]	k_{inact}/K_i [$M^{-1}s^{-1}$]	IC_{50} [nM]	k_{inact}/K_i [$M^{-1}s^{-1}$]
4	30.7 ± 1.6	1 270 000 ± 196 000	70 ± 0	45 500 ± 5 700	50 ± 0	45 000 ± 14 000
5	144.6 ± 3.3	116 000 ± 1260	50 ± 3	42 400 ± 2 400	256.7 ± 20	29 900 ± 670
6	16.4 ± 3.7	1 610 000 ± 297 000	53 ± 4	138 000 ± 50 000	9.6 ± 0.4	759 000 ± 72 000
7	26.0 ± 1.2	1 530 000 ± 54 400	64.2 ± 0.5	117 000 ± 16 500	8.15 ± 0.8	704 000 ± 73 800
8	NI[b]	ND[c]	52 000 ± 1400	ND	46 000 ± 5900	ND
9	9500 ± 300	ND	24 000 ± 300	ND	13 000 ± 100	ND
K11777	ND	552 000 ± 109 000[d]	ND	23 800 ± 840	ND	950 000 ± 170 000

[a] Data are the mean ± SD of $n = 3$ independent experiments performed in duplicate. [b] No inhibition at 20 μM. [c] Not determined. [d] Published value: 555 000.[10]

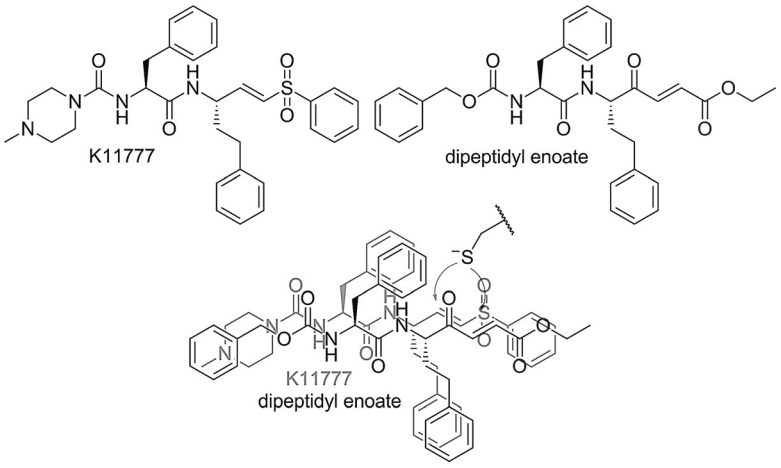


Figure 1. Michael acceptor inhibitors.

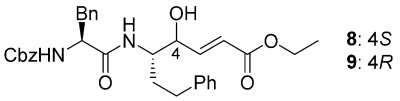
shows the best selectivity and the highest reactivity, as it displays the lowest IC_{50} and highest k_{inact}/K_i values of all compounds tested. Inhibitor **7**, with *cis* geometry at the C=C double bond, exhibits a slightly lower activity than its *trans*-isomer, **6**.

The compounds were also tested against human cysteine proteases cathepsins B and L, which, like rhodesain, belong to the papain-family proteases (Table 1). In some cases IC_{50} values were similar to those of rhodesain, but in all cases the k_{inact}/K_i value was found to be one or two orders of magnitude lower than that against rhodesain.

To confirm irreversibility of inhibition, dilution assays with rhodesain and the test compounds were carried out; K11777, a well-known irreversible vinylsulfone, was used as a positive control (Supporting Information). Inhibitors **4**–**7** were shown to display a reversible mode of inhibition at very low concentrations, and then an irreversible mode at higher concentrations.

The antitrypanosomal activity of compounds **4** and **5** against *T. b. rhodesiense* trypomastigotes was determined by a serial dilution assay. Inhibitors **4** and **5** exhibited IC_{50} values of 0.505 and 1.178 μg mL^{-1}, respectively. The two compounds were also tested for cytotoxicity, and exhibited IC_{50} values of 2.775 and 1.735 μg mL^{-1}, respectively, against L6 cells.

To determine the importance of the carbonyl group for inhibitor activity, isomeric alcohols **8** and **9** were prepared and tested against rhodesain. These compounds were synthesized via a synthetic route previously reported by our research group[11] (Supporting Information). Compound **8** did not inhibit rhodesain, and compound **9** inhibited rhodesain in the micromolar range (Table 1). These results confirm the carbonyl group as being key for the inhibitory activity of dipeptidyl enoates. Curiously, compound **9**, with opposite configuration to that of **8** at C4 is active, whereas compound **8** is not.



Docking was performed with AutoDock Vina[12] (version 1.1.2) between the X-ray crystallographic structure of rhodesain (PDB ID: 2P7U) and inhibitor **6** as ligand. Comparison between the docked conformation of **6** and the conformation of K11777 in complex with rhodesain shows inhibitor **6** to be oriented similarly to K11777 (Figure 2a).[13] The ester group of **6** is involved in interactions with Gln19, Trp184, and His162 (distances of 3.233, 3.146, and 3.204 Å, respectively) as is sulfone group of K11777. The NH group of the modified Phe residue of **6** is involved in hydrogen bonding with the carbonyl oxygen of Asp161 and the ketone oxygen of **6** ($d = 3.082$ and 2.912 Å, respectively; Figure 2b). Moreover, the NH group of Phe in compound **6** is hydrogen bonded with the carbonyl oxygen atom of Gly66 ($d = 2.890$ Å; Figure 2b).[13]

Based on the kinetics and docking data, a mode of action for these inhibitors can be suggested. Dipeptidyl enoates display a reversible mode of inhibition (Scheme 2): a rapid reversible attack of the thiolate to the ketone carbonyl to give a hemithioacetal intermediate as a reversible E·I complex could be considered.[14] An irreversible conjugate addition of the Cys25 thiolate followed by protonation of the resulting enolate by

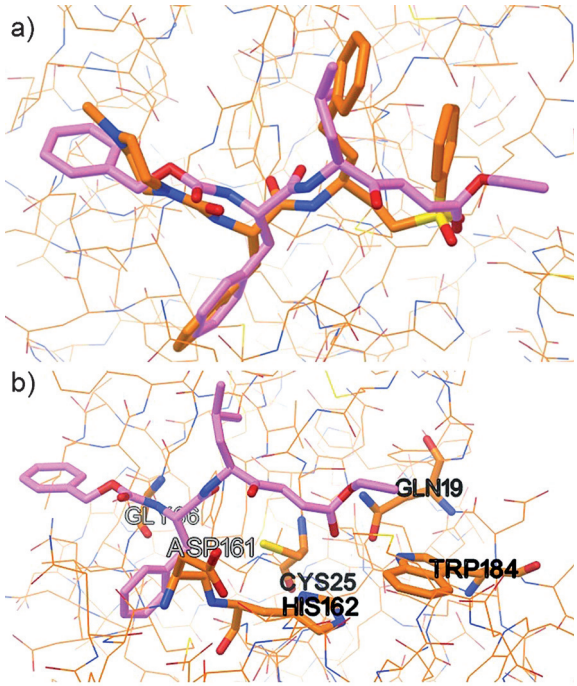
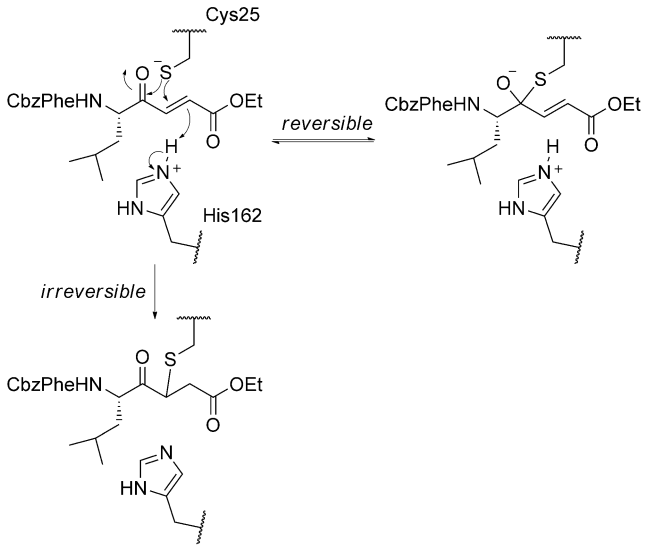


Figure 2. a) Docking pose of inhibitor **6** (magenta) superimposed with K11777 (orange) in complex with rhodesain (PDB ID: 2P7U). b) Proposed docking pose of inhibitor **6** (magenta) in complex with rhodesain.



Scheme 2. Mode of action of dipeptidyl enoates.

His162 might also take place. Regarding the regioselectivity of the attack, the activation of the ester group by interactions with Gln19, Trp184, and His162 would support attack of the α position of the ketone (Scheme 2). It is expected the *cis* compound **7** would not form a reversible complex as efficiently as the *trans*-configured **6**, but once the conjugate addition takes place, the resulting enolate would be the same for both isomers.

In summary, we report a new type of potent rhodesain inhibitor, displaying a 4-oxoenoate moiety as a warhead embedded in a dipeptidic backbone. Kinetics and docking studies suggest the inhibitors to follow two separate pathways: one reversible and other irreversible, which finally lead to an irreversibly blocked enzyme.

Experimental Section

General. ^{1}H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ (^{1}H: 7.24 ppm, ^{13}C: 77.0 ppm) solution at 30 °C on 300 or 500 MHz NMR spectrometers. Mass spectra were measured with a QTOF I (quadrupole–hexapole–ToF) mass spectrometer using an orthogonal *Z*-spray–electrospray ionization interface. IR spectra were recorded as oily films on NaCl plates on an FTIR spectrometer. Silica gel 60 (EM Science) was used for column chromatography, whereas TLC was performed on pre-coated silica plates (thickness: 0.25 mm). Unless otherwise specified, all reactions were carried out under N_2 atmosphere with magnetic stirring.

General procedure for the preparation of phosphonates: *n*-Butyllithium (1.6 M in hexanes; 1.98 mL, 19.17 mmol) was added to a solution of dimethyl methylphosphonate (2.08 mL, 19.17 mmol) in THF (20 mL) at −70 °C. The resulting mixture was stirred at −70 °C for 15 min, and then a solution of the corresponding doubly protected dipeptide (previously prepared by standard procedures; 2.40 mmol) in THF (20 mL) was added dropwise. The resulting mixture was stirred at −70 °C for 2 h and was then quenched with 10% AcOH (20 mL) and extracted with EtOAc (3×30 mL). The organic layers were washed (saturated $NaHCO_3$ solution), dried (Na_2SO_4), and concentrated. The crude oil was submitted directly to the next step without further purification.

Benzyl ((*S*)-1-(((*S*)-1-(dimethoxyphosphoryl)-2-oxo-5-phenylpentan-3-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (1). Yield: 98%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.48 (d, *J* = 7.8 Hz, 1H), 7.27–7.08 (m, 15H), 5.68 (d, *J* = 7.2 Hz, 1H), 5.07 (d, *J* = 12.6 Hz, 1H), 5.01 (d, *J* = 12.3 Hz, 1H), 4.63–4.53 (m, 2H), 3.71 (s, 3H), 3.67 (s, 3H), 3.18–2.91 (m, 2H), 2.53 (t, *J* = 7.8 Hz, 2H), 2.24–2.13 (m, 1H), 1.89–1.80 ppm (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 200.3, 170.9, 156.1, 140.7, 136.2, 129.2, 128.6, 128.4, 128.0, 127.9, 126.9, 126.0, 66.9, 58.7, 56.2, 53.1, 53.0, 38.6, 36.9, 31.9, 31.5 ppm.

Benzyl ((*S*)-1-(((*S*)-1-(dimethoxyphosphoryl)-2-oxo-5-phenylpentan-3-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (2). Yield: 89%; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.38–7.13 (m, 10H), 5.47 (d, *J* = 7.0 Hz, 1H), 5.11 (d, *J* = 12.0 Hz, 1H), 5.07 (d, *J* = 12.5 Hz, 1H), 4.61 (dq, *J* = 8.5, 4.5 Hz, 1H), 4.25 (m, 1H), 3.71 (s, 3H), 3.69 (s, 3H), 3.28 (dd, *J* = 22.6, 14.1 Hz, 1H), 3.05 (dd, *J* = 21.9, 14.1 Hz, 1H), 2.60 (m, 2H), 2.23 (m, 1H), 1.92 (m, 1H), 1.69 (m, 2H), 1.52 (m, 1H), 0.93 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 200.8, 172.9, 156.0, 140.6, 136.1, 128.4, 128.0, 127.9, 126.0, 66.8, 58.5, 53.8, 52.1, 52.0, 41.3, 38.6, 36.9, 31.8, 31.6, 24.7, 23.1 ppm.

Benzyl ((*S*)-1-(((*S*)-1-(dimethoxyphosphoryl)-5-methyl-2-oxohexan-3-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (3). Yield: 92%; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.35–7.17 (m, 10H), 5.46 (d, *J* = 7.5 Hz, 1H), 5.08 (d, *J* = 12.0 Hz, 1H), 5.04 (d, *J* = 12.5 Hz, 1H), 4.62 (dt, *J* = 10.0, 4.0 Hz, 1H), 4.50 (m, 1H), 3.74 (d, *J* = 6.5 Hz, 3H), 3.72 (d, *J* = 6.5 Hz, 3H), 3.22–3.06 (m, 3H), 2.97 (dd, *J* = 22.0, 14.0 Hz, 1H), 1.62 (ddd, *J* = 13.6, 9.4, 4.1 Hz, 1H), 1.38–1.53 (m, 3H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.85 ppm (d, *J* = 6.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 201.4, 171.0, 155.8, 136.1, 129.4, 128.7, 128.6, 128.4, 128.0, 127.1, 67.0, 57.5, 56.2, 53.1, 52.9, 39.6, 38.6, 38.0, 24.6, 23.1, 21.3 ppm.

General procedure for the preparation of dipeptidyl enoates: K_2CO_3 (1 mmol) and ethyl glyoxalate (1 mmol) were added to a stirred solution of the corresponding dipeptidyl phosphonate (1 mmol) in EtOH (7.5 mL). The resulting mixture was stirred at room temperature for 2 h and was then filtered, neutralized with AcOH, and concentrated. The crude oil was purified by silica gel chromatography (hexanes/EtOAc 7:3).

(*S*,*E*)-Ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxo-7-phenylhept-2-enoate (4). Yield: 76%; white solid (mp: 115–119 °C); $[\alpha]_D^{20} = -12.0$ ($c = 0.1$, $CHCl_3$); IR (NaCl): $\tilde{\nu} = 3619$, 3019, 2896, 2399, 1716, 1518, 1385, 1213, 1046, 928, 746, 734, 669, 627 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 7.35–7.06$ (m, 16H), 6.72 (d, $J = 16.0$ Hz, 1H), 6.50 (br s, 1H), 5.21 (br s, 1H), 5.13 (d, $J = 12.5$ Hz, 2H), 5.09 (d, $J = 12.0$ Hz, 2H), 4.84 (q, $J = 7.0$ Hz, 1H), 4.44 (m, 1H), 4.28 (q, $J = 7.5$ Hz, 2H), 3.07 (m, 2H), 2.45–2.58 (m, 2H), 2.18–2.25 (m, 1H), 1.83–1.90 (m, 1H), 1.34 ppm (t, $J = 7.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 196.3$, 170.7, 165.0, 155.9, 140.3, 136.2, 135.8, 132.7, 129.3, 128.7, 128.6, 128.5, 128.4, 128.2, 128.0, 127.1, 126.4, 67.2, 61.7, 56.9, 56.2, 38.4, 32.7, 31.2, 14.1 ppm; HRMS *m/z* calcd for $C_{32}H_{35}O_6N_2$ $[M+H]^+$: 543.2495, found: 543.2496.

(*S*,*E*)-Ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-4-methylpentanamido)-4-oxo-7-phenylhept-2-enoate (5). Yield: 80%; colorless oil; $[\alpha]_D^{20} = -14.5$ ($c = 1.1$, $CHCl_3$); IR (NaCl): $\tilde{\nu} = 3404$, 3064, 2961, 1736, 1679, 1518, 1455, 1369, 1247, 1188, 1029, 979, 764, 726, 717 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 7.33–7.07$ (m, 11H), 6.81 (m, 1H), 6.73 (d, $J = 15.9$ Hz, 1H), 5.13 (s, 2H), 4.84–4.90 (m, 1H), 4.26 (q, $J = 6.6$ Hz, 2H), 4.18 (m, 1H), 2.60 (m, 1H), 2.24 (m, 1H), 1.92 (m, 1H), 1.68 (m, 2H), 1.51 (m, 1H), 1.32 (t, $J = 7.2$ Hz, 3H), 0.94 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 196.9$, 172.0, 165.2, 156.2, 140.4, 135.8, 132.8, 128.6, 128.6, 128.5, 128.3, 128.1, 128.1, 126.4, 67.2, 61.6, 57.1, 53.6, 41.2, 32.8, 31.3, 24.7, 22.9, 22.0, 14.1 ppm; HRMS *m/z* calcd for $C_{30}H_{32}O_6N_2$ $[M+H]^+$: 509.2652, found: 509.2657.

(*S*,*E*)-Ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-7-methyl-4-oxooct-2-enoate (6). Yield: 84% for **6** and **7**; white solid (mp: 71–75 °C); $[\alpha]_D^{20} = +8.0$ ($c = 1.0$, $CHCl_3$); IR (NaCl): $\tilde{\nu} = 3618$, 3019, 2896, 2436, 2399, 1717, 1519, 1369, 1211, 1046, 929, 793, 765, 755, 747, 731, 719, 625 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 7.36–7.12$ (m, 10H), 6.80 (d, $J = 16.0$ Hz, 1H), 6.61 (br s, 1H), 5.49 (d, $J = 8.0$ Hz, 1H), 5.08 (s, 2H), 4.83 (td, $J = 9.0$, 3.5 Hz, 1H), 4.52 (m, 1H), 4.28 (q, $J = 7.0$ Hz, 2H), 3.07 (m, 2H), 1.55 (m, 2H), 1.34 (t, $J = 7.0$ Hz, 3H), 1.30 (m, 1H), 0.93 (d, $J = 5.5$ Hz, 3H), 0.86 ppm (d, $J = 6.0$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta =$ 197.1, 170.6, 165.0, 155.8, 136.2, 136.1, 132.5, 129.3, 128.6, 128.1, 127.1, 67.1, 61.5, 56.9, 56.7, 40.2, 38.2, 24.7, 23.0, 21.7, 14.1 ppm; HRMS *m/z* calcd for $C_{28}H_{35}O_6N_2Na$ $[M+Na]^+$: 517.2315, found: 517.2317.

(*S*,*Z*)-Ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-7-methyl-4-oxooct-2-enoate (7). Yellow oil; $[\alpha]_D^{20} = -28.1$ ($c = 1.2$, $CHCl_3$); IR (NaCl): $\tilde{\nu} = 3417$, 3020, 2961, 1720, 1498, 1386, 1302, 1216, 1027, 910, 771, 762, 738, 669 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 7.39–7.20$ (m, 10H), 6.43 (d, $J = 8.5$ Hz, 1H), 6.40 (d, $J = 12.0$ Hz, 1H), 6.00 (d, $J = 12.0$ Hz, 2H), 5.19 (br s, 1H), 5.10 (s, 2H), 4.77 (td, $J = 10.0$, 4.0 Hz, 1H), 4.47 (q, $J = 6.5$ Hz, 1H), 4.20 (m, 2H), 3.12 (m, 2H), 1.76 (m, 1H), 1.55 (m, 1H), 1.46 (m, 1H), 1.29 (t, $J = 7.5$ Hz, 3H), 0.92 (d, $J = 6.5$ Hz, 3H), 0.90 ppm (d, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 197.2$, 170.5, 165.1, 155.6, 139.5, 136.0, 132.5, 129.4, 129.2, 128.9, 128.5, 128.2, 128.0, 126.8, 67.3, 61.6, 56.7, 56.1, 40.4, 38.3, 24.9, 23.2, 21.8, 14.1 ppm; HRMS *m/z* calcd for $C_{28}H_{35}O_6N_2Na$ $[M+Na]^+$: 517.2315, found: 517.2318.

Acknowledgements

The authors thank Serveis Centrals d'Instrumentació Científica at Universitat Jaume I for technical support. S.R. thanks the Generalitat Valenciana for a postdoctoral research grant under the VALi+d Program. T.S. thanks the Deutsche Forschungsgemeinschaft (DFG) (SFB630) for financial support.

Keywords: dipeptidyl enoates · inhibitors · rhodesain · sleeping sickness · trypanosomiasis

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[14] In the docking studies, the respective distances between the sulfur atom of Cys25 and the ketone carbonyl group, Cα to the ketone, and Cα to the ester in compound **6** are 4.664, 4.153, and 4.504 Å.

Received: May 8, 2015
Revised: June 25, 2015
Published online on July 14, 2015