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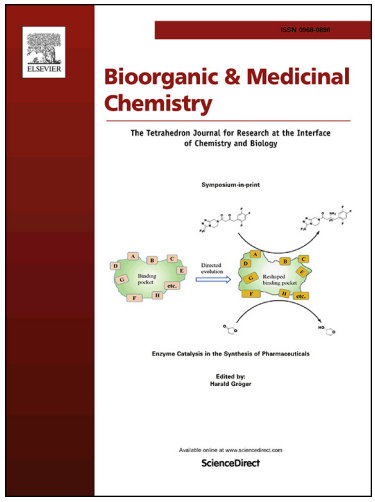

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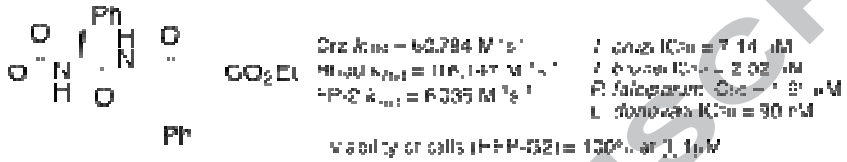

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ABSTRACT

A family of dipeptidyl enoates has been prepared and tested against the parasitic cysteine proteases rhodesain, cruzain and falcipain-2 related to sleeping sickness, Chagas disease and malaria, respectively. They have also been tested against human cathepsins B and L1 for selectivity. Dipeptidyl enoates resulted to be irreversible inhibitors of these enzymes. Some of the members of the family are very potent inhibitors of parasitic cysteine proteases displaying k_{2nd} ($M^{-1}s^{-1}$) values of seven orders of magnitude. *In vivo* antiprotozoal testing was also performed. Inhibitors exhibited IC_{50} values in the micromolar range against *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi* and even more promising lower values against *Leishmania donovanii*.

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1. Introduction

Malaria, sleeping sickness and Chagas disease are among the most important tropical diseases, and the last two are considered neglected.[1,2] Human African trypanosomiasis (HAT) or sleeping sickness, caused by the protozoan *Trypanosoma brucei*, is fatal when untreated, and current treatments are ineffective and have side effects. The cysteine protease rhodesain, being essential for the development of *T. brucei*, has been identified as an interesting target for the search of new drugs against this disease. Chagas disease is caused by the protozoan *Trypanosoma cruzi*. Approximately 7-8 million people are infected by *T. cruzi* in Central and South America, with over 100 million people at risk of infection.[1] It has recently emerged in North America and Europe as well. Benznidazole and nifurtimox are drugs currently available but both have variable efficacy and side effects.[3] The cysteine protease cruzain has been identified as a target for the search of new drugs against this disease.[4] Malaria is the most widespread and severe tropical infectious disease; in humans, it is caused by several species of the *Plasmodium* genus, with *Plasmodium falciparum* being the most dangerous and most prevalent. The cysteine protease falcipain-2 has been recognized as a potential drug target.

All three above mentioned parasitic cysteine proteases rhodesain, cruzain and falcipain-2 belong to the papain superfamily. The alignment of these three proteases with their homologous cathepsin B and L shows striking similarities at three main functional regions (see Supplementary Material)[5] despite of some other structural differences allowing the potential design of inhibitors selectivity as antiparasitic compounds.[6]

Michael acceptors are among the most interesting inhibitors of cysteine proteases. For example, K1777 a dipeptidyl vinyl sulfone is a potent irreversible inhibitor.[7]

We previously reported dipeptidyl enoates as efficient inhibitors against rhodesain,[8] and we now report a structure-activity study of dipeptidyl enoates as irreversible inhibitors against the parasitic cysteine proteases falcipain-2, cruzain and rhodesain as compared to their activity against human cathepsins B and L. As it is reported herein *in vitro* and antiprotozoal activity of these inhibitors which contain an enoate moiety at the carboxyl terminus depended very much on the residues within the peptidic framework and the protecting group of the amino terminus.

2. Results and discussion

2.1. Structure design of inhibitors

The structure of the inhibitors is a modified dipeptide having a carbon-carbon double bond conjugated with an ester at the carboxyl-terminus of the dipeptide and a protecting group at the amino-terminus (Figure 1). The conjugated double bond at the carboxyl-terminus is the warhead to be attacked by the thiolate of the cysteine. Analogues with different substituens were prepared in order to optimize the interactions at the sites S_1, S_2, S_3 and S_1'.

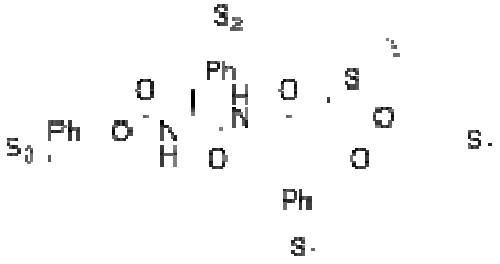
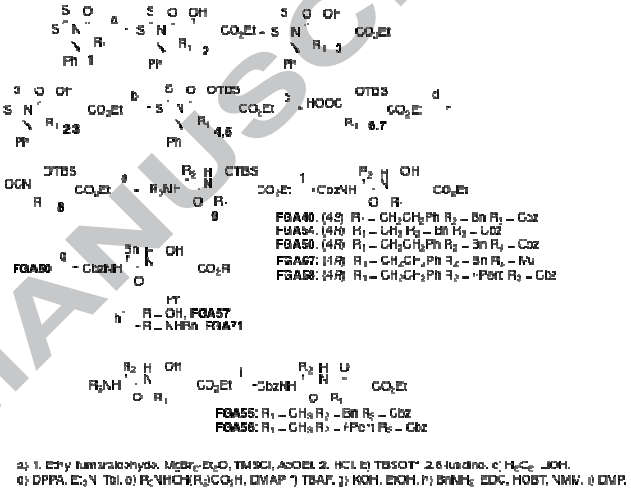


Figure 1. Schematic representation of binding of the dipeptidyl enoate inhibitors into the active site and the binding pockets.

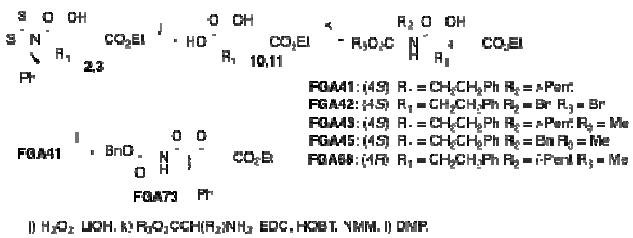
2.2. Synthesis of inhibitors

The designed inhibitors were initially prepared through a synthetic route resulting from a combination of an asymmetric Evans aldol reaction, followed by protection/deprotection steps and then a Curtius reaction to afford the corresponding isocyanate which upon coupling with corresponding *N*-protected aminoacid yielded the hydroxylic enoates FGA40, FGA54, FGA50, FGA67 and FGA58 (Scheme 1). Since the aldol reaction affords a mixture of separable isomers, epimeric inhibitors differing in the configuration of carbon-4 could also be prepared. Then, the oxidation of FGA54 afforded the ketones FGA55 and FGA56 (Scheme 1).



Scheme 1. Preparation of inhibitors through an Evans aldol/Curtius sequence.

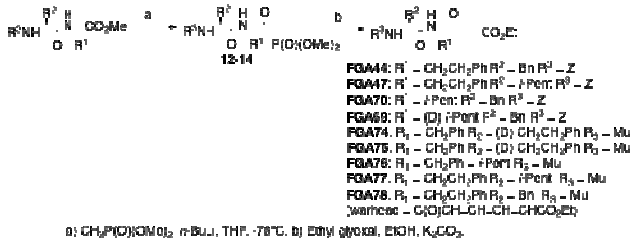
Analogues displaying a reverse sequence were also prepared by coupling the carboxylic acids resulting from chiral auxiliary removal with an aminoester (FGA41, 42, 43, 45, 68) (Scheme 2). Chemical derivatizations of these compounds afforded further members of the family. FGA41 was oxidized to give the corresponding ketone FGA73, and the ester FGA50 was hydrolized into carboxylic acid FGA57 which was subsequently derivatized into amide FGA71 (Scheme 2).



Scheme 2. Preparation of inhibitors displaying a reversed sequence.

In order to prepare more dipeptidyl enoates, the synthetic strategy was changed. A straightforward approach was applied by preparation of phosphonates derived from the corresponding

diprotected dipeptide. Then, Horner-Emmons reactions between dipeptidyl phosphonate and ethyl glyoxalate afforded the corresponding inhibitors in good yield (Scheme 3).



a) $CH_2P(O)(OMe)_2$, n-BuLi, THF, -78°C. b) Ethyl glyoxal, EtOH, K_2CO_3.

Scheme 3. Preparation of inhibitors through phosphonates.

2.3. Inhibitory activity against cysteine proteases

In the alcohol series, *in vitro* testing against cysteine proteases denote inhibitors to display a time-dependent inhibition with IC_{50} values in the micromolar range (Table 1). The most active inhibitor of this series resulted to be FGA67 displaying a morpholinyl carbonyl protecting group instead of the benzyloxy carbonyl which is part of the other compounds. Inhibitor FGA67 gave IC_{50} values between 1 and 4 μM. Interestingly, the diastereomeric inhibitors FGA50 and FGA40 differing only in the configuration of one stereocenter showed different activities. FGA50, displaying S configuration at C-4, was more active than FGA40, with *R* configured C-4 atom.

For all assayed cysteine proteases, FGA54 with an L-alanine residue at the P1 site showed similar inhibitory (slightly lower) activity than its L-homophenylalanine counterpart FGA50.

Inhibitor FGA57 having a carboxylic acid was slightly less active than the ethyl ester FGA50, and the benzylamide FGA71 was not active.

FGA41, FGA42, FGA43 and FGA45 consisting of a reverse amide bond as compared to the rest of inhibitors were not active which demonstrates the importance of the correctly oriented amide bond for these dipeptidic inhibitors.

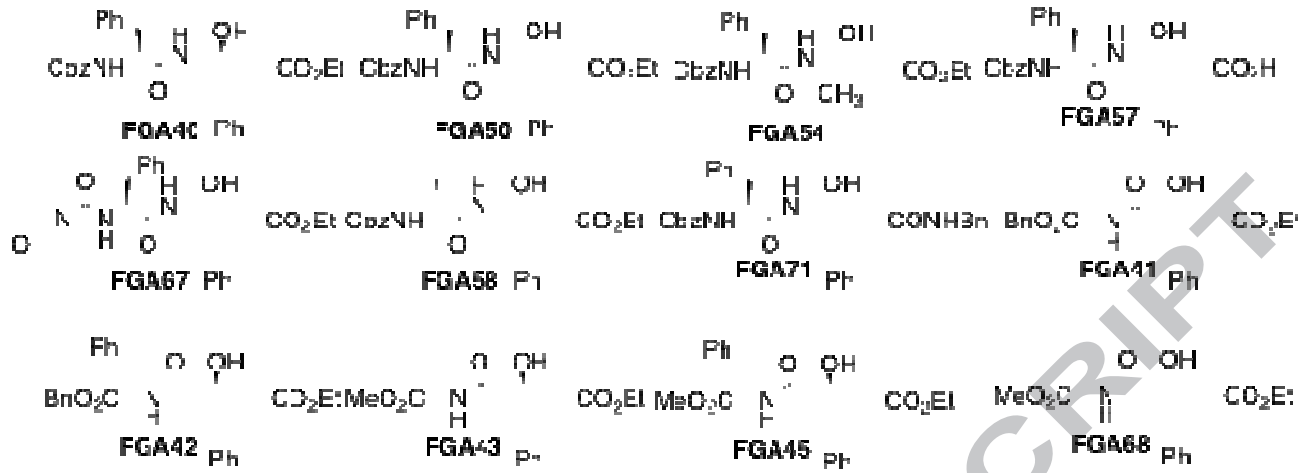


Table 1. *In vitro* inhibitory activity of compounds against cysteine proteases

	Cathepsin B			Cathepsin L			Falcipain-2			Rhodesain			Cruzain		
Comp.	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]	Inh.%[a]	IC_{50}[b]	k_{2nd}[c]	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]	Inh.%[a]	IC_{50}[b]	k_{2nd}[c]	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]
FGA40	35	52.6	14	58	47	77	22		nd	32		nd	37		
FGA41	17		nd	0		nd	5		nd	2		nd	nd		
FGA42	25		nd	0		nd	5		nd	6		nd	nd		
FGA43	0		nd	0		nd	3		nd	1		nd	nd		
FGA45	0		nd	0		nd	9		nd	4		nd	nd		
FGA50	36	23.9	800	57	13.1	1100	41	34.4	557	63	9.5	3200	18		nd
FGA54	26		nd	80	14.3	828	41	26.8	2700	63	15.1	11000	30		nd
FGA57	15		nd	47	20	882	24	75	29	24		nd	12		nd
FGA58	10		nd	14		nd	11		nd	0		nd	9		nd
FGA67	100	1.13	4200	98	2.45	1900				99	1.13	36700	95	3.75	3600
FGA68															
FGA71	9		nd	17		nd				1		nd	41		nd

[a]Inh. % at 20 μM.

[b]IC_{50} in μM units

[c]k_{2nd} in $M^{-1}s^{-1}$ units.

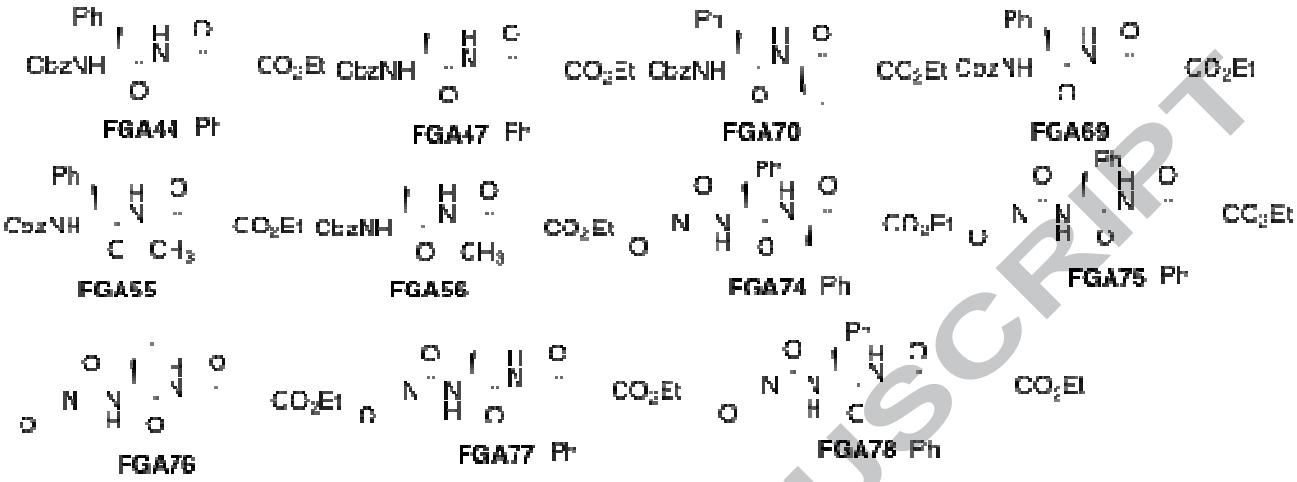
Inhibitors with a ketone group resulted to be more potent against the tested cysteine proteases than the counterparts with hydroxyl group (Table 2). The inhibitors FGA44, FGA55 and FGA75 containing a L-phenylalanine residue at P-2 site were more active than FGA47, FGA56 and FGA77 having an L-leucine residue at this position. For the P-1 site L-homophenylalanine (FGA44, FGA47, FGA74, FGA75 and FGA77) or L-leucine (FGA69 and FGA76) gave better results than L-alanine (FGA55 and FGA56). Inhibitors FGA69 and FGA 70 differing in the configuration of the leucine residue gave similar results with the L-leucine derivative being slightly more active. A similar result was observed when comparing inhibitors FGA74 and FGA75.

Inhibitors having a morpholine carbonyl group were less potent than the ones with a benzyloxy carbonyl group. For example, FGA44 displayed higher IC_{50} values than FGA75, and the kinetic constants ratio k_{2nd} was found to be higher against rhodesain; similar results were observed when comparing FGA70 with FGA76 or FGA47 with FGA77.

Interestingly, compound FGA78 having a dienoate warhead is a reversible inhibitor as opposite to the ones having an enoate moiety which are all irreversible. The (ir)reversibility of inhibition was tested by dilution assays for compounds FGA74, FGA75, and FGA78.

Tested compounds also inhibited human cathepsins B and L. Further SAR studies will be carried out in the near future by changing amino acids at P1 and P2 positions so as to improve selectivity. The structures of human and parasite cathepsins cocrystalized with inhibitors revealed sites for modification in the inhibitor to enhance selectivity for the parasite protease over its human ortholog.[9]

Table 2. *In vitro* inhibitory activity of ketone compounds against cysteine proteases



	Cathepsin B			Cathepsin L			Falcipain-2			Rhodesain			Cruzain		
Comp.	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]	Inh.%[a]	IC_{50}[b]	k_{2nd}[c]	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]	%Inh.[a]	IC_{50}[b]	k_{2nd}[c]
FGA44	100	0.07	45500	100	0.05	45300	100	0.06	17100	100	0.03	1274500	99		
FGA47	100	0.05	42400	100	0.26	29900	98	0.58	6000	98	0.145	116200	100	0.08	60800
FGA55	100	0.14	47000	100	0.08	197300	99	0.19	28300	100	0.59	6500	100	0.3	139600
FGA56	100	0.07	85500	99	0.5	9800	98	1.2	12000	99	0.8	14200	99	0.63	14400
FGA69	100	0.05	138500	100	0.01	758600	nd	nd	nd	100	0.02	1611100	100	0.02	516700
FGA70	100	0.06	116700	100	0.01	704500	nd	nd	nd	100	0.03	1533300	100	0.02	311800
FGA74	99	0.08	108400	100	> 2	nd	nd	nd	nd	99	0.15	62400	99	0.33	22000
FGA75	100	0.07	97000	100	0.18	28500	nd	nd	nd	100	0.15	142000	100	0.30	23400
FGA76	100	0.09	49500	100	0.09	52200	nd	nd	nd	100	0.11	324500	100	0.20	10600
FGA77	100	0.14	31400	100	0.21	19600	nd	nd	nd	100	0.14	120000		0.34	5600
FGA78	90	2.25	K_i=1.4 µM	99	1.49	K_i=0,78 mM	nd	nd	nd	98	0.56	K_i = 0.044 µM		> 2	0.56
K1777			23900			950000						552300[d]			

[a]Inh. % at 20 µM.

[b]IC_{50} in µM units

[c]k_{2nd} in $M^{-1}s^{-1}$ units

[d] ref. value 555000

2.4. Docking studies

Molecular modeling studies were performed in order to get an idea of the reasons for the remarkable differences in inhibition potency of keto compounds compared to the allylic alcohol compounds. Therefore, FGA50 (Ki = 9.5 µM at rhodesain) and FGA44 (Ki = 0.3 µM at rhodesain) were selected as representative examples. Docking was performed using the crystal structure of rhodesain bound to K11777 (pdb 2p7u).[10] First, non-covalent docking was carried out with FlexX/LeadIT to simulate the initial non-covalent enzyme-inhibitor complex (Figure 2).[11] The affinity of the keto compound is significantly higher (FlexX score: FGA44 –25.7, FGA50 –16.7) and the Michael system is closer to the

nucleophilic cysteine (FGA44: 3.38 Å, FGA50: 3.85 Å). The results suggest that the keto compound is attacked at the carbon next to the keto group. Furthermore, covalent docking was performed with DOCKTITE to simulate the final protein bound state (Figure 3).[12] The scores obtained do not differ significantly (Table 3), indicating no major differences once the covalent complex is formed. The main reason for the higher potency of the keto compounds may therefore be attributed to the higher non-covalent affinity resulting in a more stable non-covalent complex in which the Michael system comes closer to the nucleophilic cysteine for subsequent reaction.

Table 3. Docking results between rhodesain and compounds FGA44 and FGA50

Compound	Non-covalent docking				Covalent docking	
	FlexX score	DSX score	Distance Cys-Michael system (Å)	Affinity dG score	London dG score	DSX score
FGA44	– 25.7	– 168.9	3.38	– 6.2	– 10.6	– 130.0
FGA50	– 16.8	– 133.7	3.85	– 6.4	– 11.3	– 132.9

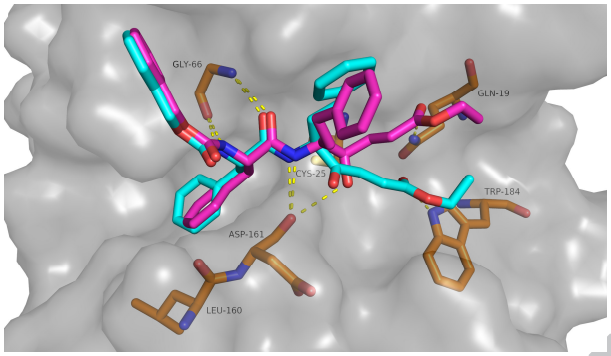


Figure 2. Overlay of non-covalent docking solutions. FGA44: cyan, FGA50: magenta.

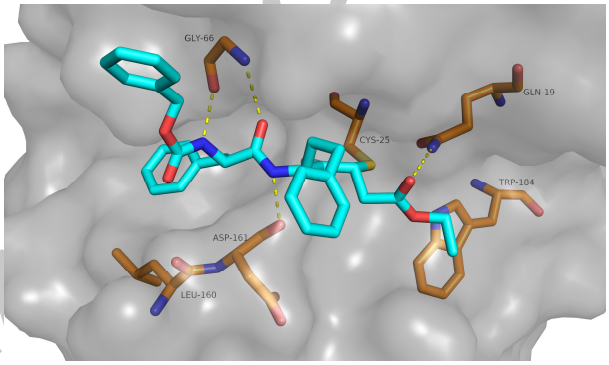


Figure 3. Covalent complex of FGA44.

2.5. Activity against Protozoa

Selected inhibitors were tested against the parasites *Trypanosoma brucei* causing Human African Trypanosomiasis, *Trypanosoma cruzi* which causes Chagas disease, and *Plasmodium falciparum* causing malaria. Some inhibitors were also tested against *Leishmania donovanii* which causes kala-azar, also called visceral leishmaniasis. As shown in Table 4, IC_{50} values ranged from nanomolar to micromolar, depending on the organism and the chemical structure of the inhibitor. Thus, FGA44 showed the best inhibitory activity against *T. brucei* (IC_{50}=0.93 µM). FGA75 was also active against *P. falciparum* (IC_{50}=6.81 µM). Inhibitor FGA47 resulted to be very active with an IC_{50} value of 1.6 µM against *P. falciparum*, and even more interestingly inhibited *L. donovanii* with an IC_{50} value of 90 nM. Such higher activity of FGA47 against *Leishmania donovanii* as compared to other tested parasites, might indicate the existence of other cysteine proteases in *L. donovanii* as potential targets.

Table 4. Antiprotozoal activity of dipeptidyl enoates (IC_{50} µM)

inhibitor	*Plasmodium falciparum*	*Trypanosoma brucei*	*Trypanosoma cruzi*	*Leishmania donovanii*
FGA-44	16.08	0.93	20.14	12.57
FGA-47	1.61	2.32	7.14	0.09
FGA-69	14.94	nd[a]	nd	nd
FGA-75	6.81	nd	nd	nd
FGA-77	17.74	nd	nd	nd

[a]nd= non-determined.

In addition, selectivity of the inhibitors was evaluated by assaying selected compounds for viability of human cells on *in vitro* culture. As seen in Figure 4, all assayed compounds allowed a 100% viability at 1µM. Thus, all these compounds performed with low cytotoxicity at low micromolar range.

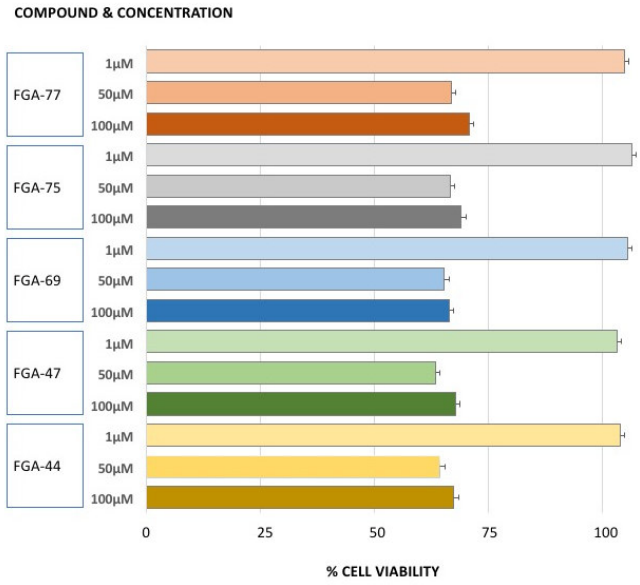


Figure 4. Cytotoxicity in human hepatoma cells HEP-G2 of selected inhibitors. HEPG2 human hepatoma cells were incubated with the inhibitors at three different concentrations (1, 50 and 100 µM). Viability of the HEPG2 human hepatoma cells after 24 h is plotted in comparison with 100% viability of control cultures in the absence of inhibitors.

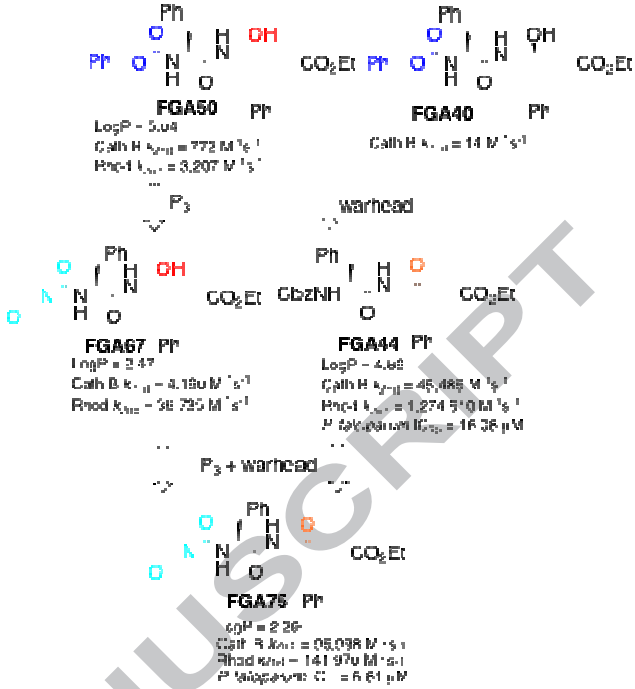


Figure 5. Optimization process of inhibitors.

3. Conclusions

An optimization study of the dipeptidyl enoates was performed by chemical structure modifications (Figure 5). Compound FGA50 displaying *R* configuration at C-3 was more active than its epimer FGA40. Two modifications of the chemical structure of FGA50 were done: the hydroxyl group of the warhead was interchanged by a ketone group resulting into FGA44, and the CBZ group at the P3 site was replaced by a morpholine carbonyl group yielding FGA67. The ketone group remarkably increased the potency, while the morpholine group lowered the calculated lipophilicity. Hence, FGA75 was prepared by combining both moieties: a ketone group at the warhead for having high activity and a morpholine carbonyl group at P3 site for a balanced lipophilicity. The resulting inhibitor FGA75 showed good activity against protozoa *Plasmodium falciparum* and low lipophilicity.

Further SAR studies will be carried out to first optimize efficacy *in vitro*, and then to provide proof of concept activity *in vivo*. For *in vitro* activity against *Leishmania*, parasites within macrophages will be used to ensure that compounds can enter the parasitophorous vacuoles within which parasites reside. This *in vitro* SAR study will then be followed by a microsomal stability study and "snapshot" pharmacokinetics prior to testing in animal models of infection. Also a preliminary toxicology study in animal models will be carried out to ensure that the compounds are safe.

4. Experimental Section

4.1. Chemistry

General. ^{1}H NMR spectra and ^{13}C NMR spectra were measured in $CDCl_3$ (1H, 7.24 ppm; 13C 77.0 ppm) solution at 30 °C on a 300 MHz or a 500 MHz NMR spectrometer. Mass spectra were measured in a QTOF I (quadrupole-hexapole-TOF) mass spectrometer with an orthogonal Z-spray-electrospray interface. IR spectra were recorded as oily films on NaCl plates on a FT-IR spectrometer. EM Science Silica Gel 60 was used for column chromatography while TLC was performed precoated plates (0.25 mm). Unless otherwise specified, all reactions were carried out under nitrogen atmosphere with magnetic stirring.

1-[(4*S*)-4-Benzyl-2-thioxo-thiazolidin-3-yl]-4-phenyl-butan-1-one 1. To an ice-bath cold solution of (*S*)-4-benzylthiazolidine-2-thione[13] (18.37 g, 87.87 mmol) and Et_3N (26.66 mL, 175.74 mmol) in CH_2Cl_2 (266 mL) was added 4-phenylbutanoyl chloride (freshly prepared from 4-phenylbutyric acid (17.31g, 105.44 mmol)). The resulting mixture was stirred at room temperature for 24 hours. Then brine was added and extracted with CH_2Cl_2 (3 x 40 mL), the organic layers were washed (brine), dried (Na_2SO_4) and concentrated. The yellow crude oil was purified through chromatography (silica-gel, hexanes/EtOAc (98:2) and (95:5)) to afford 24.76 mg (77%); $[\alpha_D^{25}]$= +157.80 (c= 2.3, $CHCl_3$). IR (NaCl) ν 3025, 2935, 2849, 1603, 1695, 1496, 1454, 1342, 1394, 1359, 1342, 1293, 1264, 1192, 1157, 1135, 1040, 893, 746, 701 cm^{-1}. ^{1}H NMR (500MHz, $CDCl_3$) δ 7.16-7.34 (10H, m), 5.33 (1H, ddd, J= 4.0, 7.5 and 11.5 Hz), 3.38 (1H, ddd, J= 6.0, 9.0 and 17.0Hz), 3.33 (1H, dd, J= 7.5 and 11.5Hz), 3.13-3.20 (2H, m), 3.01 (1H, dd, J= 10.5 and 13.0 Hz), 2.84 (1H, d, J= 11.5 Hz), 2.68 (2H, t, J= 7.0 Hz). ^{13}C NMR (125MHz, $CDCl_3$) δ 201.06, 173.73, 141.53, 136.56, 129.44, 128.88, 128.53, 128.36, 127.19, 125.96, 68.53, 37.93, 36.79, 35.11, 31.90, 26.43 ppm.. HRMS *m/z* calcd. for $C_{20}H_{21}ONS_2Na$ $[M+Na^+]$: 378.0962, found: 378.0970; *m/z* calcd. for $C_{20}H_{21}ONS_2K$ $[M+K^+]$: 394.0702, found: 394.0724.

General experimental procedure for the aldol reaction.[14]

To $MgBr_2 \cdot Et_2O$ (weighed in a dry-box) (230 mg, 0.89 mmol) were added sequentially a solution of compound 3 (2.43 g, 6.85 mmol) in EtOAc (mL), a solution of (*E*)-ethyl 3-formylacrylate (1.75 g, 13.70 mmol) in EtOAc (mL), and then freshly distilled triethylamine (2.1 ml, 15.07 mmol) and trimethylsilyl chloride (1.47 ml, 11.65 mmol). The resulting yellowish suspension was stirred under nitrogen atmosphere at room temperature for 12 days. Then the reaction mixture was filtered through a pad of silica-gel using ethyl ether and then concentrated under vacuum. Then the resulting orange oil was solved in THF (180 mL) and treated with hydrochloric acid (1M aqueous solution) (4 mL). Then solid $NaHCO_3$ was added until no more gas was evolved. Then extracted with CH_2Cl_2 (3 x 40 mL), dried (Na_2SO_4) and concentrated. The yellow crude oil was purified through chromatography (silica-gel, CH_2Cl_2/Et_2O (20:1)) to afford 2.15 g of a yellow oil (yield 65%, over consumed starting material).

Spectroscopical data for *anti* aldol: (2*E*,4*S*,5*S*)-5-[(4*S*)-4-Benzyl-2-thioxo-thiazolidine-3-carbonyl]-4-hydroxy-7-phenyl-hept-2-enoic acid ethyl ester 2.

$[\alpha_D^{29}]$ = +113.31(c= 3.7, $CHCl_3$). IR (NaCl) ν 3483, 3027, 2980, 1716, 1603, 1496, 1455, 1368, 1342, 1263, 1164, 1137, 1038, 982, 912, 865, 747, 701 cm^{-1}.^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05-7.25 (10H, m), 6.84 (1H, dd, J = 15.5 and 4.0 Hz), 6.03 (1H, dd, J= 15.5, 1.0 Hz), 5.14 (1H, ddd, J= 4.5, 7.5 and 11.0 Hz), 4.72 (1H, m), 4.46 (1H, m), 4.10 (2H, q, J= 7.5 Hz), 3.25 (1H, dd, J = 7.5 and 11.0 Hz,), 3.04 (1H, dd, J = 4.5 and 13.5 Hz), 2.91 (1H, dd, J = 10.0 and 13.5 Hz), 2.76 (2H, d, J = 11.5 Hz), 2.57 (1H, m), 2.12 (1H, m), 2.03 (1H, m), 1.18 (3H, t, J= 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.85, 175.77, 166.09, 147.74, 141.15, 136.14, 129.39, 128.88, 128.43, 128.40, 127.27, 126.10, 121.89, 71.25, 68.44, 60.59, 48.01, 36.71, 32.87, 32.40, 30.45, 14.16 ppm. HRMS *m/z* calcd. for $C_{26}H_{29}O_4NS_2Na$ [M+Na^+]: 506.1436, found: 506.1429; *m/z* calcd. for $C_{26}H_{29}O_4NS_2K$ [M+K^+]: 522.1175, found: 522.1180.

Spectroscopical data for *syn* aldol: (2*E*,4*R*,5*S*)-5-[(4*S*)-4-Benzyl-2-thioxo-thiazolidine-3-carbonyl]-4-hydroxy-7-phenyl-hept-2-enoic acid ethyl ester 3.

$[\alpha_D^{29}]$ = +55.0 (c= 1.9, $CHCl_3$). IR (NaCl) ν 3468, 3027, 2929, 1714, 1604, 1496, 1455, 1368, 1342, 1264, 1162, 1038, 911, 745, 701 cm^{-1}.^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10-7.28 (10H, m), 6.83 (1H, dd, J= 15.5 and 4.5 Hz), 6.03 (1H, dd, J= 15.5, 1.0 Hz), 5.30 (1H, ddd, J= 4.0, 7.5 and 11.5 Hz), 5.05 (1H, dq, J= 4.5 Hz), 4.58 (1H, m), 4.13 (2H, q, J= 7.5 Hz), 3.31 (1H, dd, J= 7.5 and 12.0 Hz), 3.08 (1H, dd, J= 3.5 and 13.5 Hz), 2.94 (1H, dd, J = 10.5 and 13.5 Hz), 2.85 (2H, d, J= 11.5 Hz), 2.54-2.67 (2H, m), 2.46 (1H, d, J= 4.0 Hz), 2.12-2.18 (m, 2H), 1.82-1.89 (m, 2H), 1.22 (3H, t, J= 7.0 Hz). ^{13}C NMR (125 MHz, CCl_3) δ 202.06, 175.03, 166.14, 146.61, 141.59, 136.32, 129.48, 129.42, 129.29, 129.00, 128.49, 128.47, 127.37, 126.11, 122.12, 72.02, 69.05, 60.63, 48.21, 36.98, 33.69, 32.00, 28.84, 14.26 ppm. HRMS *m/z* calcd. for $C_{26}H_{29}O_4NS_2Na$ [M+Na^+]: 506.1436, found: 506.1441.

General experimental procedure for the protection of aldols.

To a -70ºC cold solution of aldol (1.81 g, 3.75 mmol) in CH_2Cl_2 (38 mL) was added 2,6-lutidine (1.31 mL, 11.25 mmol) and then TBSOTf (2.15 mL, 9.37 mmol). The resulting mixture was stirred at –70 ºC for 6h. Then quenched with saturated aqueous $NaHCO_3$ solution, and extracted with CH_2Cl_2 (3 x 40 mL), dried (Na_2SO_4) and concentrated. The crude was purified through chromatography (silica-gel, hexane/EtOAc (8:2) to afford a yellow oil.

Spectroscopical data for *anti* silylated aldol: (2*E*,4*S*,5*S*)-5-[(4*S*)-4-Benzyl-2-thioxo-thiazolidine-3-carbonyl]-4-(*tert*-butyl-dimethyl-silanyloxy)-7-phenyl-hept-2-enoic acid ethyl ester 4.

(yield= 98%). $[\alpha_D^{21}]$ = +92.65 (c= 5.8, $CHCl_3$). IR (NaCl) ν 3063, 3027, 2930, 2857, 1716, 1659, 1604, 1496, 1455, 1367, 1341, 1230, 1159, 1038, 984, 837, 779, 746, 701, 667 cm^{-1}. ^{1}H NMR (500MHz, $CDCl_3$) δ 7.15-7.37 (10H, m), 6.93 (1H, dd, J = 7.0 and 15.5 Hz), 6.01 (1H, d, J = 15.5 Hz), 5.37 (1H, ddd, J = 3.5, 7.0 and 11.0 Hz), 5.21 (1H, m), 4.58 (1H, t, J= 6.5 Hz), 4.22 (2H, t, J= 7.0 Hz), 3.31 (1H, dd, J= 7.5 and 11.5 Hz), 3.20 (1H, dd, J= 3.5 and 13.5 Hz), 3.04 (1H, dd, J= 11.0 and 13.5 Hz), 2.85 (1H, d, J= 11.0 Hz), 2.76 (1H, m), 2.64 (1H, m), 2,10 (1H, m), 1.90 (1H, m), 1.31 (3H, t, J = 7.0 Hz), 0.92 (9H, m), 0.08 (3H, s), 0.04 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.44, 174.18, 165.76, 147.52, 141.60, 136.39, 129.35, 128.80, 128.29, 128.26, 127.12, 125.86, 122.40, 74.27, 68.93, 60.47, 49.32, 36.81, 33.04, 31.55, 30.67, 25.61, 17.86, 14.11, -4.27, -5.05 ppm. HRMS *m/z* calcd. for $C_{32}H_{43}O_4NS_2SiNa$ [M+Na^+]: 620.2301, found: 620.2283.

Spectroscopical data for *syn* silylated aldol: (2*E*,4*R*,5*S*)-5-[(4*S*)-4-Benzyl-2-thioxo-thiazolidine-3-carbonyl]-4-(*tert*-butyl-dimethyl-silanyloxy)-7-phenyl-hept-2-enoic acid ethyl ester 5.

(yield= 94%). $[\alpha_D^{20}]$ = +112.1 (c= 1.5, $CHCl_3$). IR (NaCl) ν 3063, 3027, 2954, 2930, 2857, 1718, 1659, 1604, 1496, 1471, 1455, 1365, 1341, 1262, 1193, 1161, 1135, 1083, 1038, 983, 939, 883, 838, 779, 747, 701, 667 cm^{-1}. ^{1}H NMR (500MHz, $CDCl_3$) δ 7.15-7.36 (10H, m), 6.94 (1H, dd, J= 5.5 and 15.5 Hz), 5.93 (1H, d, J= 15.5 Hz), 5.26 (1H, ddd, J = 4.0, 7.0 and 11.0 Hz), 5.07 (1H, dq, J = 5.0 Hz), 4.55 (1H, t, J = 5.0 Hz), 4.20 (2H, q, J= 6.5 Hz), 3.30 (1H, dd, J= 7.0 and 11.5 Hz), 3.17 (1H, dd, J= 4.0 and 13.0 Hz), 3.01 (1H, dd, J= 11.0 and 13.0 Hz), 2.84 (1H, d, J= 11.5 Hz), 2.61-2.73 (2H, m), 2.24 (1H, m), 1.87 (1H, m), 1.30 (3H, t, J= 7.0 Hz), 0.89 (9H, s), 0.01 (3H, s), 0.00 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.57, 173.62, 166.04, 147.67, 141.61, 136.45, 129.45, 129.42, 129.36, 129.10, 128.40, 128.37, 128.35, 128.32, 127.18, 125.91, 121.72, 73.46, 69.23, 60.47, 49.68, 36.67, 33.52, 31.78, 29.07, 25.73, 25.67, 18.03, 14.20, -4.61, -5.21 ppm. HRMS *m/z* calcd. for $C_{32}H_{43}O_4NS_2SiNa$ [M+Na^+]: 620.2301, found: 620.2310; calcd. for $C_{32}H_{43}O_4NS_2SiK$ [M+K^+]: 636.2040, found: 636.2079.

General experimental procedure for the removal of the chiral auxiliary.

To an ice-bath cold solution of protected aldol (2.24 g, 3.75 mmol) in THF-H_2O (3:1) (67.5 mL) was added H_2O_2 (30% v/v) (2.32 mL, 22.5 mmol) and then a solution of LiOH (180 mg, 7.50 mmol) in H_2O (1.8 mL). The resulting mixture was stirred cold with an ice-bath for 2 h. Then quenched with a saturated aqueous solution of Na_2SO_3 and $NaHCO_3$ and stirred for 30 min. Then extracted with CH_2Cl_2 (3 x 40 mL), dried (Na_2SO_4) and concentrated. The crude oil was purified through chromatography (acidified silica-gel, hexanes/EtOAc (7:3)) to afford an oil.

(2*E*,4*S*,5*S*)-4-(tert-Butyl-dimethyl-silanyloxy)-5-phenethyl-hex-2-enedioic acid 1-ethyl ester 6.

(yield= 88%). $[\alpha_D^{21}]$ = -9.88 (c= 8.6, $CHCl_3$). IR (NaCl) ν 3028, 2930, 2858, 2649, 1944, 1710, 1660, 1605, 1497, 1472, 1455, 1391, 1369, 1260, 1163, 1092, 1035, 1006, 982, 938, 837, 778, 748, 700, 670 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.58 (1H, s), 7.14-7.27 (5H, m), 6.82 (1H, dd, J= 6.0 and 15.5 Hz), 5.96 (1H, d, J= 1.5 and 15.5 Hz), 4.50 (1H, t, J = 1.0 and 7.0 Hz), 4.18 (2H, m), 2.71 (1H, m), 2.61 (2H, m), 1.87 (1H, m), 1.76

(1H, m), 1.27 (3H, t, J= 7.0 Hz), 0.86 (9H, s), 0.03 (3H, s), 0.00 (3H, s). ^{13}C NMR (125MHz, $CDCl_3$) δ 178.51, 166.00, 146.81, 141.04, 128.53, 128.50, 126.17, 122.83, 73.01, 60.65, 52.03, 33.68, 29.31, 25.71, 18.06, 14.24, -4.37, -4.50 ppm. HRMS *m/z* calcd. for $C_{22}H_{34}O_5SiNa$ [M+Na$^+$]: 429.2073, found: 429.2099.

(2*E*,4*R*,5*S*)-4-(*tert*-Butyl-dimethyl-silanyloxy)-5-phenethyl-hex-2-enedioic acid 1-ethyl ester 7.

(yield= 83%). [α_D^{20}] = -10.35 (c= 1.3, $CHCl_3$). IR (NaCl) ν 3028, 2957, 2931, 2859, 1712, 1660, 1604, 1497, 1472, 1455, 1391, 1369, 1260, 1165, 1125, 1084, 1037, 1006, 979, 939, 837, 778, 748, 699, 671 cm^{-1}. ^{1}H NMR (500MHz, $CDCl_3$) δ 10.58 (1H, s), 7.15-7.28 (5H, m), 6.87 (1H, dd, J= 5.5 and 15.5 Hz), 5.97 (1H, d, J= 15.5 Hz), 4.60 (1H, t, J= 4.5 Hz), 4.19 (2H, m), 2.77 (1H, m), 2.55 (2H, m), 2.05 (1H, m), 1.82 (1H, m), 1.28 (3H, t, J= 7.0 Hz), 0.88 (9H, s), 0.03 (3H, s), 0.00 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.48, 166.12, 147.41, 141.22, 128.48, 128.46, 126.10, 122.21, 72.68, 60.58, 51.02, 33.74, 28.10, 25.75, 18.10, 14.23, -4.47, -4.52 ppm. HRMS *m/z* calcd. for $C_{22}H_{34}O_5SiNa$ [M+Na$^+$]: 429.2072, found: 429.2108; *m/z* calcd. for $C_{22}H_{34}O_5SiK$ [M+K$^+$]: 445.1813, found: 445.1866.

General experimental procedure for the conversion of the carboxylic acid into isocianate.

To a solution of carboxylic acid (660 mg, 1.63 mmol) in toluene (13 mL) was added 4 molecular sieves (196 mg), Et_3N (249 µL, 1.79 mmol) and then diphenylphosphorylazide (401 µL, 1.79 mmol). The resulting mixture was stirred at room temperature for 30 min. Then was quenched with a saturated aqueous solution of hydrogen carbonate and extracted with Et_2O (3 x 30 mL), dried (Na_2SO_4) and concentrated. The crude oil was purified through chromatography (silica-gel, hexanes/EtOAc (8:2) and (7:3)) to afford an oil.

(2*E*,4*S*,5*S*)-4-(*tert*-Butyl-dimethyl-silanyloxy)-5-isocyanato-7-phenyl-hept-2-enoic acid ethyl ester 8.

(yield= 54%). [α_D^{20}] = -16.05 (c= 4.5, $CHCl_3$). IR (NaCl) ν 3028, 2956, 2931, 2859, 2253, 1783, 1722, 1660, 1604, 1497, 1472, 1367, 1260, 1175, 1128, 1038, 982, 838, 778, 700 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13-7.27 (5H, m), 6.84 (1H, dd, J= 5.0 and 15.5 Hz), 6.00 (1H, dd, J= 15.5 and 1.5 Hz), 4.23 (1H, m), 4.19 (2H, m), 3.22 (1H, m), 2.83 (1H, m), 2.62 (1H, m), 1.82 (2H, q, J= 7.5 Hz), 1.27 (3H, t, J= 7.0 Hz), 0.88 (9H, s), 0.03 (3H, s), 0.00 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.89, 146.34, 140.51, 128.58, 128.46, 126.24, 122.95, 73.86, 60.59, 59.21, 34.55, 32.34, 25.69, 18.07, 14.19, -4.40, -4.99 ppm. HRMS *m/z* calcd. for $C_{22}H_{33}O_4NSiNa$ [M+Na$^+$]: 426.2077, found: 426.2071.

Ethyl (4*S*,5*S*,*E*)-5-(((*S*)-1-(benzyloxy)-4-methyl-1-oxopentan-2-yl)carbamoyl)-4-hydroxy-7-phenylhept-2-enoate (FGA41).

(yield = 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13-6.89 (10H, m), 6.60 (1H, dd, J= 4.4 and 15.6 Hz), 6.12 (1H, d, J = 8.3 Hz), 5.86 (1H, dd, J= 15.6 and 1.6 Hz), 4.94 (1H, d, J = 12.3 Hz), 4.90 (1H, d, J = 12.3 Hz), 4.44 (1H, td, J = 9.0 and 4.6 Hz), 4.25 (1H, m), 3.93 (2H, q, J = 7.1 Hz), 2.49-2.43 (1H, m), 2.25-2.31 (1H, m), 2.16-2.20 (1H, m), 1.80-1.88 (1H, m), 1.51-1.58 (1H, m), 1.46-1.40 (1H, m), 1.35-1.30 (1H, m) 1.03 (3H, t, J= 7.1 Hz), 0.70 (6H, d, J = 4.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 173.0, 166.2, 147.1, 141.0, 135.3, 128.6, 128.5, 128.4, 128.3, 126.2, 122.2, 72.2, 67.3, 60.5, 51.4, 51.2, 40.7, 33.4, 30.7, 25.0, 22.8, 21.6, 14.2 ppm.

Ethyl (4*S*,5*S*,*E*)-5-(((*S*)-1-(benzyloxy)-1-oxo-3-phenylpropan-2-yl)carbamoyl)-4-hydroxy-7-phenylhept-2-enoate (FGA42).

(yield = 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43-7.12 (11H, m), 7.01-6.98 (4H, m), 6.80 (1H, dd, J= 4.5 and 16.1 Hz), 6.21 (1H, d, J = 8.3 Hz), 6.06 (1H, dd, J= 16.1 and 1.7 Hz), 5.20 (1H, d, J = 12.0 Hz), 5.12 (1H, d, J = 12.0 Hz), 4.92 (1H, m), 4.32 (1H, m), 4.12 (2H, q, J = 7.1 Hz), 3.70 (1H, m), 3.10 (dd, J = 13.5, 6.5 Hz, 1H), 3.00 (dd, J = 13.5, 8.5 Hz, 1H), 2.52-2.32 (2H, m), 2.22-2.24 (1H, m), 2.12-2.20 (1H, m), 1.80-1.87 (1H, m), 1.26 (3H, t, J= 7.1 Hz) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 171.5, 166.2, 147.1, 140.9, 135.6, 129.1, 128.8, 128.7, 128.6, 128.5, 128.4, 127.3, 126.2, 122.2, 72.0, 67.6, 60.5, 53.2, 51.0, 37.5, 33.1, 30.8, 14.2 ppm

Ethyl (4*S*,5*S*,*E*)-5-(((*S*)-1-(methyloxy)-4-methyl-1-oxopentan-2-yl)carbamoyl)-4-hydroxy-7-phenylhept-2-enoate (FGA43).

(yield = 51%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30-7.15 (5H, m), 6.81 (1H, dd, J= 4.4 and 15.6 Hz), 6.10 (1H, dd, J= 15.6 and 1.6 Hz), 4.60 (1H, m), 4.35 (1H, m), 4.21 (1H, q, J = 7.0 Hz), 3.65 (3H, s), 2.72 (1H, m), 2.61 (1H, m), 2.25 (1H, m), 2.15 (1H, m), 1.80 (1H, m), 1.55 (1H, m), 1.49 (1H, m), 1.10 (3H, t, J= 7.0 Hz), 0.70 (6H, d, J = 4.4 Hz).

Ethyl (4*S*,5*S*,*E*)-5-(((*S*)-1-(methyloxy)-1-oxo-3-phenylpropan-2-yl)carbamoyl)-4-hydroxy-7-phenylhept-2-enoate (FGA45).

(yield = 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35-6.89 (10H, m), 6.82 (1H, dd, J= 4.5 and 16.1 Hz), 6.10 (1H, m), 6.08 (1H, d, J= 16.1 Hz), 4.85 (1H, m), 4.30 (1H, m), 4.10 (2H, q, J = 7.1 Hz), 3.75 (1H, m), 3.70 (s, 3H), 3.10 (dd, J = 13.5, 6.5 Hz, 1H), 3.00 (dd, J = 13.5, 8.5 Hz, 1H),2.55-2.34 (2H, m), 2.21 (1H, m), 2.05 (1H, m), 1.75 (1H, m), 1.25 (3H, t, J= 7.1 Hz) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 172.0, 166.2, 147.2, 140.9, 135.7, 129.0, 128.8, 128.5, 128.4, 127.4, 126.2, 122.2, 72.1, 60.5, 53.1, 52.6, 50.8, 37.6, 33.1, 30.9, 14.2 ppm.

Ethyl (*E*)-5-(((*S*)-1-(benzyloxy)-4-methyl-1-oxopentan-2-yl)carbamoyl)-4-oxo-7-phenylhept-2-enoate (FGA73).

To an ice-bath cold solution of ethyl (4*S*,5*S*,*E*)-5-(((*S*)-1-(benzyloxy)-4-methyl-1-oxopentan-2-yl)carbamoyl)-4-hydroxy-7-phenylhept-2-enoate (FGA41) (310 mg, 0.63 mmol) in dichloromethane (6.25 mL) was added Dess-Martin periodinane (517 mg, 1.22 mmol). The resulting mixture was stirred at room temperature for 1h. Then saturated aqueous solution of sodium bicarbonate with some sodium thiosulfate crystals (10 mL) was added and stirred for additional 15 min. The mixture was extracted with Et_2O (3 x 20 mL), dried over $MgSO_4$ and concentrated under reduce pressure. The product was purified through chromatography (silica-gel, hexane/ethyl acetate (8:2)) to afford a white solid.

(yield = 55%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45-7.11 (10H, m), 6.72 (1H, d, J= 4.5 Hz), 6.60 (1H, d, J= 4.5 Hz), 6.45 (1H, m), 6.10 (1H, m), 5.72 (1H, d, J= 16.1 Hz), 5.66 (1H, d, J= 16.1 Hz), 4.70 (1H, m), 4.25 (2H, m), 3.70 (1H, m), 2.55-3.25 (3H, m), 2.20 (1H, m), 1.47-1.75 (3H, m), 1.26 (3H, t, J= 7.1 Hz) 0.82 (6H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.1, 196.7, 172.5, 172.2, 171.8, 167.7, 167.6, 166.4, 165.0, 162.4, 140.2, 137.6, 137.3, 134.3, 132.8, 128.8, 128.7, 128.6, 128.4, 128.3, 126.5 126.4, 125.4, 105.1, 67.3, 67.2, 61.5, 60.8, 58.3, 51.1, 51.0, 41.6, 36.9, 33.2, 28.3, 25.0, 22.8, 22.7, 22.1, 21.8, 14.3, 14.1 ppm.

(2*E*,4*R*,5*S*)-4-(*tert*-Butyl-dimethyl-silanyloxy)-5-isocyanato-7-phenyl-hept-2-enoic acid ethyl ester 9.

(yield= 60%). $[\alpha_D^{20}]$ = -79.56 (c= 10.5, $CHCl_3$). IR (NaCl) ν 3028, 2956, 2931, 2859, 2254, 1817, 1783, 1722, 1660, 1604, 1497, 1472, 1367, 1260, 1175, 1126, 1095, 1036, 1007, 982, 838, 779, 749, 700, 671 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 7.13-7.27 (5H, m), 6.86 (1H, dd, J= 5.0 and 15.5 Hz), 5.97 (1H, d, J= 15.5 Hz), 4.54 (1H, t, J= 5.5 Hz), 4.16 (2H, m), 2.75 (1H, m), 2.51-2.60 (2H, m), 2.07 (1H, m), 1.88 (1H, m), 1.26 (3H, t, J= 7.0 Hz), 0.88 (9H, s), 0.03 (3H, s), 0.00 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.89, 146.98, 140.88, 128.55, 128.42, 126.22, 122.59, 72.25, 60.62, 52.56, 33.46, 28.56, 25.77, 18.12, 14.22, -4.30, -4.50 ppm. HRMS *m/z* calcd. for $C_{22}H_{33}O_4NSiNa$ $[M+Na^+]$: 426.2077, found: 426.1997; *m/z* calcd. for $C_{22}H_{33}O_4NSiK$ $[M+K^+]$: 442.1816, found: 442.1780.

General experimental procedure for the peptidic coupling.

To an ice-bath cold solution of the isocianate (394 mg, 0.98 mmol) in CH_2Cl_2 (6 mL) was added a solution of Cbz-L-Phe (352 mg, 1.18 mmol) in CH_2Cl_2 (2 mL) and then a solution of 4-DMAP (16 mg, 0.13 mmol) in CH_2Cl_2 (2 mL). The resulting mixture was stirred cold with an ice-bath for 1.5 h and then at room temperature for 6 h. Then quenched with brine, extracted with CH_2Cl_2 (3 x 30mL), dried (Na_2SO_4) and concentrated. The crude was purified through chromatography (silica-gel, hexane/EtOAc, (7:3) to afford an orange oil

(2E,4S,5S)-5-[(2S)-2-Benzyloxycarbonylamino-3-phenyl-propionylamino]-4-(tert-butyl-dimethyl-silanyloxy)-7-phenyl-hept-2-enoic acid ethyl ester 10.

(yield= 79%). $[\alpha_D^{20}]$ = -14.31 (c= 1.7, $CHCl_3$). IR (NaCl) ν 3322, 2929, 2858, 1719, 1659, 1535, 1497, 1455, 1366, 1260, 1174, 1130, 1031, 837, 778, 746, 698 cm^{-1}. ^1H NMR (500MHz, $CDCl_3$) δ 7.07-7.35 (15H, m), 6.53 (1H, dd, J = 4.5 and 15.5 Hz), 5.83 (1H, d, J = 15.5 Hz), 5.21 (1H, s), 5.07 (2H, s), 4.31 (1H, q, J = 7.0 Hz), 4.17 (3H, m), 3.94 (1H, m), 3.04 (2H, d, J= 7.5 Hz), 2.52 (2H, m), 1.86 (1H, m), 1.45 (1H, m), 1.26 (3H, t, J= 7.0 Hz, 0.86 (9H, s), 0.03 (3H, s), 0.00 (3H, s) ppm. ^{13}C NMR (125MHz, $CDCl_3$) δ 170.74, 166.04, 155.94, 146.69, 141.32, 136.36, 136.13, 129.28, 128.84, 128.56, 128.49, 128.44, 128.25, 128.11, 127.25, 126.03, 122.12, 72.22, 67.20, 60.47, 56.62, 53.36, 38.36, 32.49, 31.70, 25.82, 18.08, 14.27, -4.92, -5.15 ppm. HRMS *m/z* calcd. for $C_{38}H_{50}O_6N_2SiNa$ $[M+Na^+]$: 681.3336, found:681.3384.

(2*E*,4*R*,5*S*)-5-[(2*S*)-2-Benzyloxycarbonylamino-3-phenyl-propionylamino]-4-(*tert*-butyl-dimethyl-silanyloxy)-7-phenyl-hept-2-enoic acid ethyl ester 11.

(yield= 63%). $[\alpha_D^{20}]$ = -6.3 (c= 10.5, $CHCl_3$). IR (NaCl) ν 3303, 3064, 3030, 2955, 2930, 2858, 1720, 1659, 1538, 1497, 1472, 1455, 1390, 1368, 1260, 1174, 1132, 1107, 1040, 981, 838, 778, 747, 699, 671 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 7.08-7.41 (15H, m), 6.87 (1H, dd, J = 4.0 and 16.0 Hz), 6.19 (1H, dd, J = 1.5 and 16.0 Hz), 5.24 (1H, s), 5.03 (2H, s), 4.36 (1H, m), 4.20 (2H, q, J = 7.0 Hz), 4.07 (1H, m), 3.13 (1H, dd, J = 6.0 and 14.0 Hz), 3.04 (1H, m), 2.55 (2H, m), 1.82 (1H, m), 1.70 (1H, m), 1.27 (3H, t, J = 7.0 Hz), 0.89 (9H, s), 0.06 (3H, s), 0.01 (3H, s). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.48, 166.00, 156.10, 146.97, 141.64, 136.43, 135.94, 129.43, 128.73, 128.47, 128.41, 128.31, 128.27, 128.21, 128.10, 127.05, 125.99, 122.38, 73.80, 67.21, 60.52, 56.30, 53.67, 37.49, 32.39, 30.08, 25.78, 18.08, 14.21, -4.57, -5.13 ppm

General experimental procedure for the deprotection.

To an ice-bath cold solution of silylated peptide (419 mg, 0.77 mmol) in THF (8 mL), was added tetra-*n*-butyl ammonium fluoride (1M in THF) (2.70 mL, 2.70 mmol). The resulting mixture was stirred cold with an ice-bath for 5.5h. Then was concentrated and the resulting crude was purified by chromatography (Hexanes/AcOEt, (1:1) and (1:2)) to afford a white solid.

(2*E*,4*S*,5*S*)-5-[(2*S*)-2-Benzyloxycarbonylamino-3-phenyl-propionylamino]-4-hydroxy-7-phenyl-hept-2-enoic acid ethyl ester (FGA40).

(yield= 77%). mp= 141-143 ºC. $[\alpha_D^{20}]$ = -16.27 (c= 2.7, $CHCl_3$). IR (NaCl) ν 3438, 3309, 3029, 2927, 1690, 1642, 1532, 1495, 1455, 1385, 1369, 1302, 1258, 1219, 1190, 1135, 1115, 1041, 971, 911, 874, 750, 700, 673 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.09-7.35 (15H, m), 6.74 (1H, dd, J= 4.5 and 15.5 Hz), 6.31 (1H, s), 6.04 (1H, dd, J= 2.0 and 15.5 Hz), 5.42 (1H, s), 4.38 (1H, q, J= 7.2Hz), 4.23 (1H, td, J= 1.5 and 4.2 Hz), 4.17 (2H, q, J= 6.9Hz), 3.89 (1H, m), 3.01 (2H, m), 2.57 (2H, m), 1.93 (1H, m), 1.75 (1H, m), 1.26 (3H, t, J= 7.0Hz) ppm. ^{13}C NMR (75MHz, $CDCl_3$) δ 171.82, 166.22, 156.10, 146.72, 141.18, 136.26, 136.05, 129.26, 129.04, 128.96, 128.82, 128.56, 128.53, 128.37, 128.27, 128.12, 127.20, 126.10, 122.19, 72.35, 67.25, 60.53, 56.57, 54.08, 38.29, 32.32, 32.25, 14.23 ppm. HRMS *m/z* calcd. for $C_{32}H_{36}O_6N_2Na$ $[M+Na^+]$: 567.2471, found: 567.2482; *m/z* calcd. for $C_{32}H_{36}O_6N_2K$ $[M+K^+]$: 583.2210, found: 583.2203.

(2*E*,4*R*,5*S*)-5-[(2*S*)-2-Benzyloxycarbonylamino-3-phenyl-propionylamino]-4-hydroxy-7-phenyl-hept-2-enoic acid ethyl ester (FGA50).

(yield= 78%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.04-7.36 (15H, m), 6.75 (1H, dd, J = 3.5 and 15.5 Hz), 6.00 (1H, dd, J = 2.0 and 15.5 Hz), 5.84 (1H, s), 5.28 (2H, s), 5.07 (2H, s), 4.32 (1H, m), 4.16 (1H, q, J = 7.5 Hz), 4.01 (1H, m), 3.07 (1H, dd, J = 6.0 and 14.0 Hz), 2.99 (1H, dd, J = 8.0 and 13.5 Hz), 2.51 (2H, m), 1.72 (1H, m), 1.60 (1H, m), 1.24 (1H, t, J = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.7, 166.2, 156.1, 145.6, 141.1, 136.4, 136.0, 129.3, 128.9, 128.6, 128.5, 128.4, 128.2, 127.3, 126.1, 122.5, 73.1, 67.4, 60.6, 56.8, 53.9, 38.5, 32.4, 30.5, 14.2 ppm.

Ethyl (4*R*,5*S*,*E*)-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-hydroxyhex-2-enoate (FGA54).

(yield= 69%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.15-7.40 (10H, m), 6.65 (1H, dd, J = 3.5 and 15.5 Hz), 6.48 (1H, m), 6.05 (1H, dd, J = 2.0 and 15.5 Hz), 5.75 (1H, s), 5.12 (1H, d, J = 16.0 Hz), 5.05 (1H, d, J = 16.0 Hz), 4.30 (1H, m), 4.12 (1H, q, J = 7.5 Hz), 4.01 (1H, m), 3.01 (2H, m), 1.21 (1H, t, J = 7.0 Hz), 0.95 (3H, d = 6.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.2, 166.2, 156.1, 146.0, 136.4, 136.0, 129.3, 128.7, 128.5, 128.2, 128.0, 127.1, 122.1, 72.8, 67.1, 60.5, 56.6, 49.5, 38.9, 21.0, 14.2 ppm. HRMS *m/z* calcd. for $C_{25}H_{31}O_6N_2$ $[M+H^+]$: 455.2182, found: 455.2179.

Ethyl (4*R*,5*S*,*E*)-4-hydroxy-5-((*S*)-2-(morpholine-4-carboxamido)-3-phenylpropanamido)-7-phenylhept-2-enoate (FGA 67).

(yield= 99%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.05-7.45 (10H, m), 6.80 (1H, dd, J = 3.5 and 15.5 Hz), 6.10 (1H, dd, J = 2.0 and 15.5 Hz), 5.68 (1H, s), 4.72 (1H, m), 4.27 (1H, s), 4.30 (2H, q, J = 7.5 Hz), 4.01 (1H, m), 3.10-3.40 (6H, m), 2.55 (1H, m), 2.47 (1H, m), 1.70-1.55 (2H, m), 1.25 (1H, t, J = 7.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 166.2, 157.2, 146.3, 141.2, 137.0, 129.3, 128.4, 128.2, 128.2, 126.8, 125.8, 121.9, 73.0, 66.2, 60.3, 56.1, 53.8, 43.9, 38.6, 32.3, 30.5, 14.1 ppm.

Ethyl (4*R*,5*S*,*E*)-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-4-methylpentanamido)-4-hydroxy-7-phenylhept-2-enoate (FGA58).

(yield = 84%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.21-7.45 (m, 10H), 6.90 (1H, dd, J = 3.0 and 15.5 Hz), 6.65 (1H, m), 6.15 (1H, dd, J = 2.5 and 15.5 Hz), 5.32 (m, 1H), 5.10 (m, 2H), 4.30 (m, 1H), 4.21 (2H, q, J = 7.5 Hz), 3.75 (m, 1H), 2.60 (m, 2H), 1.75 (m, 1H), 1.60 (m, 1H), 1.45 (m, 1H), 1.25 (t, J = 7.5 Hz, 3H), 0.90 (m, 6H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.5, 166.4, 161.4, 156.5, 146.0, 141.1, 136.2, 128.6, 128.1, 126.1, 122.5, 73.7, 67.2, 60.5, 54.3, 53.9, 41.2, 32.3, 31.3, 25.6, 24.5, 22.7, 14.2 ppm. HRMS *m/z* calcd. for $C_{29}H_{39}O_6N_2$ [M+H$^+$]: 511.2803, found: 511.2801.

Ethyl (*S*,*E*)-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxohex-2-enoate (FGA55).

(yield= 43%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20-7.45 (10H, m), 7.20 (1H, d, J = 15.5 Hz), 6.80 (1H, d, J = 15.5 Hz), 6.65 (1H, m), 5.35 (1H, m), 5.15 (2H, s), 4.74 (1H, m), 4.48 (1H, m), 4.25 (2H, q, J = 7.5 Hz), 3.05 (3H, m), 1.22 (1H, t, J = 7.0 Hz), 1.20 (3H, d = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.7, 170.6, 165.0, 155.9, 136.1, 135.6, 133.0, 129.6, 129.3, 129.2, 128.9, 128.8, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.4, 127.2, 76.6, 67.2, 61.6, 56.2, 53.3, 45.6, 38.6, 17.3, 14.1 ppm. HRMS *m/z* calcd. for $C_{25}H_{29}O_6N_2$ [M+H$^+$]: 453.2026, found: 453.2025.

Ethyl (*S*,*E*)-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-4-methylpentanamido)-4-oxohex-2-enoate (FGA56).

(yield= 87%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30-7.45 (5H, m), 7.12 (1H, d, J = 15.5 Hz), 6.80 (1H, d, J = 15.5 Hz), 6.70 (1H, m), 5.20 (2H, s), 4.25 (2H, q, J = 7.5 Hz), 4.20 (1H. m), 1.35-1.60 (3H, m), 1.25 (1H, t, J = 7.5 Hz), 0.90 (6H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.2, 171.8, 165.0, 156.4, 136.2, 135.7, 133.0, 128.6, 128.3, 128.1, 76.6, 67.2, 61.6, 53.4, 41.5, 24.7, 22.9, 22.0, 17.3, 14.2 ppm. HRMS *m/z* calcd. for $C_{22}H_{31}O_6N_2$ [M+H$^+$]: 419.2177, found: 419.2180.

(4*R*,5*S*,*E*)-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-hydroxy-7-phenylhept-2-enoic acid (FGA57)

To an ice-bath cold solution of (2*E*,4*R*,5*S*)-5-[(2*S*)-2-benzyloxycarbonylamino-3-phenyl-propionylamino]-4-hydroxy-7-phenyl-hept-2-enoic acid ethyl ester (FGA50) (65 mg, 0.12 mmol) in a mixture of THF:H_2O (5 mL) was added KOH 1M in EtOH (240 µL, 0.24 mmol). The mixture was stirred for 2h at 0ºC and overnight at room temperature. The THF was removed under reduced pressure and 5 mL of cold water were added. $KHSO_4$ 1M aqueous solution was added until pH = 1 and the mixture was extracted using EtAcO. The organic phase was dried over $MgSO_4$ and concentrated. The product was recrystallized from a mixture of EtAcO-hexane.

(yield= 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12-7.27 (15H, m), 6.81 (1H, dd, J = 3.5 and 15.5 Hz), 6.05 (1H, dd, J = 2.0 and 15.5 Hz), 5.12 (2H, s), 4.45 (1H, m), 4.12 (1H, m), 3.85 (1H, m), 3.13 (1H, dd, J = 6.0 and 14.0 Hz), 2.92 (1H, dd, J = 8.0 and 13.5 Hz), 2.65 (1H, m), 2.47 (1H, m), 1.83 (1H, m), 1.75 (1H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.8, 172.4, 168.2, 156.8, 147.9, 141.8, 137.2, 136.8, 128.9, 128.2, 128.1, 128.0, 127.9, 127.8, 127.5, 127.3, 126.4, 125.4, 121.8, 72.7, 66.1, 56.6, 53.0, 37.7, 31.7, 31.1 ppm. HRMS *m/z* calcd. for $C_{30}H_{33}O_6N_2$ [M+H$^+$]: 517.2339, found: 517.2338.

Benzyl ((*S*)-1-(((3*S*,4*R*,*E*)-7-(benzylamino)-4-hydroxy-7-oxo-1-phenylhept-5-en-3-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (FGA71)

To an ice-bath cold solution of the (4*R*,5*S*,*E*)-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-hydroxy-7-phenylhept-2-enoic acid (FGA57) (49 mg, 0.095 mmol) in a mixture of dry CH_2Cl_2 (2 mL) and DMF (1 mL) was added subsequently HOBT (13 mg, 1eq), EDCI hydrochloride (18 mg, 1 eq), NMM (47 µL, 4.5 eq) and benzylamine (12 µL, 1.2 eq). The resulting mixture was stirred cold with an ice-bath for 1.5 h and then at room temperature for 6 h. Then quenched with brine, extracted with EtOAc (3 x 30mL), dried (Na_2SO_4) and concentrated to afford the product.

(yield= 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.48 (1H, t, J = 4 Hz), 7.90 (1H, d, J = 8 Hz), 7.50 (1H, d, J = 7.9 Hz), 7.10-7.35 (20H, m), 6.68 (1H, dd, J = 3.5 and 15.5 Hz), 6.12 (1H, dd, J = 2.0 and 15.5 Hz), 5.20 (1H, m), 4.95 (2H, s), 4.32 (3H, m), 4.12 (1H, m), 3.75 (1H, m), 2.45-2.77 (2H, m), 1.82 (1H, m), 1.65 (1H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 165.1, 156.4, 143.6, 142.6, 139.9, 138.8, 137.5, 129.7, 128.8, 128.7, 128.4, 128.1, 127.9, 127.6, 127.1, 126.6, 126.1, 124.7, 72.8, 65.6, 53.0, 42.5, 37.9, 32.3, 32.0 ppm. HRMS *m/z* calcd. for $C_{37}H_{39}O_5N_3Na$ [M+Na$^+$]: 628.2787, found: 628.2775.

General experimental procedure for the preparation of phosphonates

To a -70 ºC cold solution of dimethyl methylphosphonate (2.08 mL, 19.17 mmol) in THF (20 mL) was added *n*-butyl lithium (1.6 M in hexanes) (1.98 mL, 19.17 mmol). The resulting mixture was stirred cooled at -70 ºC for 15 min and then a solution of the corresponding diprotected dipeptide (already prepared by standard procedures) (2.40 mmol) in THF (20 mL) was added dropwise. The resulting mixture was stirred at -70 ºC for 2 h and then was quenched with 10% acetic acid (20 mL) and extracted with AcOEt (3 x 30 mL), the organic layers were washed (saturated sodium bicarbonate solution), dried (Na_2SO_4) and concentrated. The crude oil was directed submitted to the next step without any further purification.

Spectroscopic data for phosphonates:

Benzyl ((*S*)-1-(((*S*)-1-(dimethoxyphosphoryl)-2-oxo-5-phenylpentan-3-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate 12. (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 (m, 1H) ppm; ^{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 13. (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 (m, 6H) ppm; ^{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 14. (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 (d, J = 6.5 Hz, 3H) ppm; ^{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 experimental procedure for the preparation of dipeptidyl enoates

To a stirred solution of the corresponding dipeptidyl phosphonate (1 mmol) in ethanol (7.5 mL) was added potassium carbonate (1 mmol) and ethyl glyoxalate (1 mmol). The resulting mixture was stirred at room temperature for 2 h and then was filtered off, neutralized using acetic acid and concentrated. The crude oil was purified through silica gel chromatography (hexanes/ethyl acetate, 7/3).

Spectroscopic data for dipeptidyl enoates:

(*S*,*E*)-ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxo-7-phenylhept-2-enoate (FGA44). (yield = 76%). White solid (mp 115-119 ºC). $[\alpha]_D^{20}$ -12.0 (c= 0.1, $CHCl_3$). IR (NaCl) ν 3619, 3019, 2896, 2399, 1716, 1518, 1385, 1213, 1046, 928, 746, 734, 669, 627 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500MHz) δ 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 (t, J = 7.5 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 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 (FGA47). (yield = 80%). Colourless oil. $[\alpha]_D^{20}$ = -14.5 (c= 1.1, $CHCl_3$). IR (NaCl) ν 3404, 3064, 2961, 1736, 1679, 1518, 1455, 1369, 1247, 1188, 1029, 979, 764, 726, 717 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 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 (m, 6H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 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 $C_{29}H_{37}O_6N_2$ [M+H$^+$]: 509.2652, found: 509.2657.

(*R*,*E*)-Ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-7-methyl-4-oxooct-2-enoate (FGA70). (yield = 84 % for 6 and 7) White solid (mp 71-75 ºC). $[\alpha]_D^{20}$ = +8.0 (c= 1.0, $CHCl_3$). IR (NaCl) ν 3618, 3019, 2896, 2436, 2399, 1717, 1519, 1369, 1211, 1046, 929, 793, 765, 755, 747, 731, 719, 625 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 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 (d, J = 6.0 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 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*,*E*)-Ethyl 5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-7-methyl-4-oxooct-2-enoate (FGA69). Yellow oil. $[\alpha]_D^{20}$ = -28.1 (c= 1.2, $CHCl_3$). IR (NaCl) ν 3417, 3020, 2961, 1720, 1498, 1386, 1302, 1216, 1027, 910, 771, 762, 738, 669 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 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 (d, J = 6.5 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 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: 517.2317.

Ethyl (*R*,*E*)-5-((*S*)-2-(morpholine-4-carboxamido)-3-phenylpropanamido)-4-oxo-7-phenylhept-2-enoate (FGA 74).

(yield= 34%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.00-7.41 (10H, m), 6.88 (1H, d, J = 16.0 Hz), 6.00 (1H, d, J = 16.0 Hz), 5.20 (1H, m), 4.70-4.81 (2H, m), 4.51 (1H, m), 4.40 (2H, q, J = 7.5 Hz), 3.70 (5H, m), 3.30 (5H, m), 3.20 (2H, m), 2.56 (2H, m), 2.32 (1H, m), 1.80 (1H, m), 1.26 (3H, t, J = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 200.8, 172.2, 165.6, 157.1, 141.2, 136.6, 129.8, 128.4, 127.1, 126.3, 66.5, 61.4, 57.8, 55.6, 44.0, 38.5, 32.6, 31.4, 14.0 ppm. HRMS *m/z* calcd. for $C_{29}H_{35}O_6N_3Na$ [M+Na$^+$]: 544.2424, found: 544.2425.

Ethyl (*S*,*E*)-5-((*S*)-2-(morpholine-4-carboxamido)-3-phenylpropanamido)-4-oxo-7-phenylhept-2-enoate (FGA75).

(yield= 78%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12-7.37 (10H, m), 7.26 (1H, d, J = 16.0 Hz), 6.45 (1H, d, J = 16.0 Hz), 5.20 (1H, m), 4.70-4.81 (2H, m), 4.51 (1H, m), 4.50 (2H, q, J = 7.5 Hz), 3.65 (5H, m), 3.30 (5H, m), 3.20 (2H, m), 2.62 (2H, m), 2.20 (1H, m), 1.80 (1H, m), 1.27 (3H, t, J = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.5, 196.5, 171.9, 164.8, 156.9, 141.8, 140.4, 136.7, 136.0, 132.5, 129.3, 128.7, 128.6, 128.5, 128.4, 127.0, 126.3, 66.3, 61.2, 57.0, 55.8, 43.7, 38.7, 33.0, 31.5, 13.8 ppm. HRMS *m/z* calcd. for $C_{29}H_{35}O_6N_3Na$ [M+Na$^+$]: 544.2424, found: 544.2426.

Ethyl (*S*,*E*)-7-methyl-5-((*S*)-4-methyl-2-(morpholine-4-carboxamido)pentanamido)-4-oxooct-2-enoate (FGA76).

(yield= 43%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (1H, d, J = 16.0 Hz), 6.90 (1H, m), 6.80 (1H, d, J = 16.0 Hz), 5.05 (1H, m), 4.78 (1H, m), 4.45 (1H, m), 4.54 (2H, q, J = 7.5 Hz), 4.48 (1H, m), 3.72 (5H, m), 3.32 (5H, m), 1.30-1.75 (7H, m), 1.30 (3H, t, J = 7.0 Hz), 1.25 (2H, m), 0.75 (12H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 198.0, 173.2, 165.3, 157.8, 136.3, 132.1, 66.4, 61.6, 56.1, 52.6, 44.1, 42.0, 40.4, 24.9, 23.2, 22.8, 22.4, 21.9, 14.2 ppm. HRMS *m/z* calcd. for $C_{22}H_{37}O_6N_3Na$ [M+Na$^+$]: 462.2580, found: 462.2574.

Ethyl (*S*,*E*)-5-((*S*)-4-methyl-2-(morpholine-4-carboxamido)pentanamido)-4-oxo-7-phenylhept-2-enoate (FGA77).

(yield= 78%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25-7.40 (6H, m), 6.70 (1H, d, J = 16.0 Hz), 5.20 (1H, m), 4.75 (1H, m), 4.40 (1H, m), 4.20 (2H, m), 3.72 (5H, m), 3.30 (5H, m), 2.60 (2H, m), 2.20 (1H, m), 1.71 (1H, m), 1.60 (3H, m), 1.32 (3H, t, J = 7.0 Hz), 0.75 (6H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.1, 173.8, 173.7, 165.1, 165.0, 157.5, 140.4, 136.1, 132.3, 132.2, 128.5, 128.4, 126.3, 66.4, 61.4, 57.1, 53.0, 44.1, 41.3, 32.5, 31.4, 24.8,

22.8, 22.3, 14.1 ppm. HRMS *m/z* calcd. for $C_{26}H_{37}O_6N_2Na$ [M+Na$^+$]: 510.2581, found: 510.2580.

Ethyl (*S*,2*E*,4*E*)-7-((*S*)-2-(morpholine-4-carboxamido)-3-phenylpropanamido)-6-oxo-9-phenylnona-2,4-dienoate (FGA78).

To a stirred solution of the corresponding dimethyl ((3R,6S)-6-(morpholine-4-carboxamido)-2,5-dioxo-3-phenethyl-7-phenylheptyl)phosphonate (0.67 mmol) in ethanol (7.5 mL) was added potassium carbonate (0.67 mmol) and ethyl (E)-4-oxobut-2-enoate (341 mg, 2.66 mmol) solved in EtOH (10 mL). The resulting mixture was stirred at room temperature for 2 h and then was filtered off, neutralized using acetic acid and concentrated. The crude oil was purified through silica gel chromatography using ethyl acetate to afford 151 mg of the desired product.

(yield= 41%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12-7.37 (12H, m), 7.26 (1H, dd, J = 16.0, 15.0 Hz), 6.45 (1H, dd, J = 16.0, 15.0 Hz), 5.20 (1H, m), 4.60-4.76 (2H, m), 4.40 (1H, m), 4.24 (2H, q, J = 7.5 Hz), 3.82 (4H, m), 3.30 (4H, m), 3.22 (2H, m), 2.50 (2H, m), 2.25 (2H, m), 1.27 (3H, t, J = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.7, 180.3, 171.7, 165.6, 157.1, 140.8, 140.6, 140.3, 136.9, 136.7, 131.8, 130.0, 129.3, 128.7, 128.6, 128.5, 128.4, 127.0, 126.2, 66.3, 60.9, 56.4, 55.4, 38.6, 33.1, 14.1 ppm. HRMS *m/z* calcd. for $C_{31}H_{36}O_6N_3Na$ [M+Na$^+$]: 570.2580, found: 570.2579.

4.2. Biological evaluation

4.2.1 Enzyme assays

The inhibitory activity was tested as reported previously.[8,15-24] Dilution assays were performed as described previously.[18]

4.2.2 Protozoa assays

Antimalarial drug activity assay

P. falciparum strain Dd2 (clone MRA-150; Malaria Research and Reference Reagent Resource Center: http://www.mr4.org) was maintained in continuous culture following the protocol previously described.[22] The culture media consisted of standard RPMI 1640 (Sigma-Aldrich) supplemented with 0.5 % Albumax I (Gibco), 100 µM hypoxanthine (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), 12.5 µg/mL gentamicine (Sigma-Aldrich), and 25 mM $NaHCO_3$ (Sigma-Aldrich), and incubated in 5% CO_2 at 37 ºC in tissue culture flasks (Iwaki). Growth progress was monitored by microscopy in thin blood smears stained with Wright's eosin methylene blue solution (Merck), using the freely available Plasmoscore software[26] to determine parasitaemia values. A detailed description of *P. falciparum* culture and synchronization methods used in this work has been reported previously.[25]

Drug activity was assayed by the PicoGreen microfluorimetric DNA-based assay monitoring parasite growth inhibition at different drug concentrations.[27] PicoGreen (P7589) was purchased from Invitrogen and diluted as indicated by the manufacturer in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Synchronized rings from stock cultures were used to test serial dilutions of the inhibitors in 96-well culture microplates. Thus, 150 µL of parasites at 2% hematocrit and 1% parasitemia were allowed to grow for 48 h in 5% CO_2 at 37 ºC. The parasites were then centrifuged at 600×g for 10 min and resuspended in saponin (0.15%, wt/vol, in phosphate-buffered saline (PBS)) to lyse the erythrocytes and release the malaria parasites. To eliminate all traces of hemoglobin, the pellet was washed by the addition of 200 µL of PBS followed by centrifugation at 600 × g. The washing step was repeated twice to ensure complete removal of hemoglobin. Finally, pellets were resuspended in 100 µL of PBS. A 100-µL volume of PicoGreen diluted in TE was added to each well. Plates were incubated for 30 to 60 min in the dark, and the fluorescence intensity was measured at 485-nm excitation and 528-nm emission. Growth inhibition defining the half maximal inhibitory concentration (IC_{50}) was calculated as previously described.[23] Upon treatment, the parasite morphology was evaluated by microscopic analysis of thin blood smears stained with Wright's stain. Smears from drug-free cultures were used as a control.

In vitro cell viability assay

HEPG2 human hepatoma cells were cultured 24 h in triplicate in 96-well flat-bottomed microplates in DMEM Glutamax (GIBCO) growth medium in a final volume of 100 µL per well. Cells grown in the presence of medium alone were used as controls. To evaluate the potential cytotoxic effect of the G6PD inhibitors, one set of plates received serial dilutions from 0.2 to 2 mM of each inhibitor. Cells were seeded during 24 h and 48 h in triplicate in 96-well flat-bottomed microplates at a density of 5 × 10^3 cells per well in 100 µL growth medium. Treatment and control in growth medium was performed after 24 h and during further 24 h incubation in a final volume of 200 µL (100 µL cells + 100 µL medium with or without treatment). Following treatment, cell viability was determined by means of the colorimetric EZ4U (Biomedica, Vienna, Austria) according to the manufacturer instructions, and absorbance at 490/610 nm was measured spectrophotometrically in a Cary 50 BIO Microplate Reader 50MPR (Varian).

Activity against Trypanosoma brucei rhodesiense STIB900.

This stock was isolated in 1982 from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions[28] Minimum Essential Medium (50 µl) supplemented with 25 mM HEPES, 1g/l additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. Then 4x10^3 bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µl was added to each well and the plate incubated at 37 °C under a 5 % CO_2 atmosphere for 70 h. 10 µl Alamar Blue (resazurin, 12.5 mg in 100 ml double-distilled water) was then added to each well and incubation continued for a further 2–4 h.[29] Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC50 values were calculated by linear regression[30] from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, Sunnyvale, CA, USA). Melarsoprol (Arsobal Sanofi-Aventis, received from WHO) is used as control.

Activity against T. cruzi.

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtitre plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h the medium was removed and replaced by 100 µl per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene.[31] After 48 h the medium was removed from the wells and replaced by 100 µl

fresh medium with or without a serial drug dilution of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml. After 96 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 µl) was added to all wells. A color reaction developed within 2–6 h and could be read photometrically at 540 nm. Data were analyzed with the graphic programme Softmax Pro (Molecular Devices), which calculated IC_{50} values by linear regression[30] from the sigmoidal dose inhibition curves. Benznidazole is used as control (IC50 0.5±0.2µg/ml).

Activity against L. donovani axenic amastigotes.
Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium[32] at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. One hundred microlitres of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtitre plates. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. After 70 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µl of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water)[33] were then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (= inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated.

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Supplementary Material