Development of Peptidomimetics with a Vinyl Sulfone Warhead as Irreversible Falcipain-2 Inhibitors

Roberta Ettari,*[,†] Emanuela Nizi,[‡] Maria Emilia Di Francesco,[‡] Marie-Adrienne Dude,[§] Gabriele Pradel,[§] Radim Vičík,[||] Tanja Schirmeister,[||] Nicola Micale,[†] Silvana Grasso,[†] and Maria Zappalà[†]

Dipartimento Farmaco−Chimico, University of Messina, Viale Annunziata, 98168 Messina, Italy, Department of Medicinal Chemistry, IRBM, MRL Rome, Via Pontina Km30.600, 00040 Pomezia, Roma, Italy, Research Center for Infectious Diseases, University of Würzburg, Röntgenring 11, D-97070 Würzburg, Germany, and Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

Received September 12, 2007

This paper describes the synthesis of a new class of peptidomimetic cysteine protease inhibitors based on a 1,4-benzodiazepine scaffold and on an electrophilic vinyl sulfone moiety. The former was introduced internally to a peptide sequence that mimics the fragment D-Ser-Gly; the latter was built on the P1-P1′ site and reacts as a classical "Michael acceptor", leading to an alkylated enzyme by irreversible addition of the thiol group of the active site cysteine. The introduction of the vinyl sulfone moiety has been accomplished by olefin cross-metathesis, a powerful tool for the formation of carbon−carbon double bonds. New compounds **2**–**3** have been proven to be potent and selective inhibitors of falcipain-2, a cysteine protease isolated from *Plasmodium falciparum*, displaying antiplasmodial activity.

Introduction

Malaria is one of the most important infectious diseases in the world, it is prevalent in more than 90 countries, and about 40% of the world's population is at risk of malaria transmission. It is estimated that there are some 350–500 million cases resulting in over 1 million deaths each year.[1] Malaria is caused by protozoan erythrocytic parasites of the Plasmodium genus, with *P. falciparum* being the most dangerous and widespread disease-causing species. Due to the increasing resistance of malaria parasites to antimalarial drugs, the lack of highly effective vaccines, and inadequate control of mosquito vectors, the problem is growing, especially in the developing world. Thus, new approaches to drug development are needed.[2]

Falcipain-2 (FP-2) of *P. falciparum* is a papain-family (clan CA, family C1) cysteine protease that plays an important role in the parasitic life-cycle by degrading erythrocyte proteins, most notably hemoglobin.[3] The protozoan parasite relies on hemoglobin hydrolysis to supply amino acids for protein synthesis and to maintain osmotic stability. The binding of hemoglobin to FP-2 is pH-modulated, and normally the enzyme processes hemoglobin within the acidic food vacuole.

During the late trophozoite and schizont stages, FP-2 is also involved in the degradation of erythrocyte-membrane skeletal proteins, including ankyrin and the band 4.1 protein.[4] This activity displays a pH-optimum in the range of 7.0–7.5 and is thought to contribute to destabilization of the erythrocyte membrane, leading to host cell rupture and release of the mature merozoites. The autoproteolytic processing of its own precursor at neutral pH has been suggested as a third function of FP-2.[5] FP-2 is synthesized during the trophozoite stage as a membrane-bound proenzyme comprising 484 amino acid residues.[6] The proenzyme is transported to the food vacuole through the endoplasmatic reticulum/Golgi system, and during this process the N-terminal 243 residues containing the membrane anchor are proteolytically removed. Hence, FP-2 is actually considered the prime target for discovery of novel antimalarial drugs.

Several peptide-based FP-2 inhibitors have been identified.[7] However, their utility as therapeutic agents is limited due to their susceptibility to protease degradation and their poor absorption through cell membranes. Efforts to alleviate these problems have been undertaken[8] and ranged from the simple replacement of natural with unnatural amino acids[9,10] to the insertion of scaffold devoid of any peptide bond.[11] In such a way, it was possible to increase the lipophilicity and the activity to the required target as well as to improve the robustness of the drug to the enzymatic degradation. A common strategy to achieve an improvement in selectivity is to lock a defined conformation of the peptide pharmacophore into a rigid scaffold,[11] which could mimic the secondary structures of proteins as well.

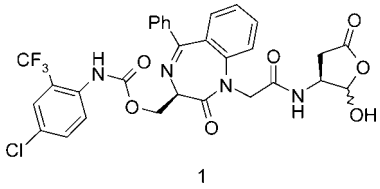
The β-turn is a structural motif that has been postulated in most cases for the biologically active form of linear peptides, and several scaffolds mimicking β-turns have been reported in recent years.[12] With its similar molecular dimensions, the benzodiazepine (BZD) nucleus has been shown to be a good mimetic of a number of β-turn types.[13] Coupled with the fact that, as a drug class, BZDs are known to have good oral bioavailability and to be well tolerated, we recently combined these observations to synthesize β-turn mimetics FP-2 inhibitors with the BZD core as a template.[14] This new class of peptidomimetics is based on a benzo[1,4]diazepin-2-one scaffold introduced internally to a peptide sequence that mimics the fragment D-Ser-Gly and based on a C-terminal aspartyl aldehyde building block that inhibits the enzyme by forming a reversible covalent bond at the active site. All peptidomimetic derivatives (e.g., **1**, Chart 1) provided significant inhibition of FP-2 with IC_{50} in the range 8–26 μM.

In order to further investigate the structural requirements of this class of inhibitors, we envisaged the synthesis of novel peptidomimetic compounds, that is derivatives **2** and **3** (Chart 2), whereby the P1-aspartyl aldehyde of derivative **1**, the most

* To whom correspondence should be addressed. Phone: +39 090 6766466. Fax: +39 090 355613. E-mail: rettari@pharma.unime.it.
† University of Messina.
‡ IRBM, MRL Rome.
§ Research Center for Infectious Diseases, University of Würzburg.
|| Institute of Pharmacy and Food Chemistry, University of Würzburg.

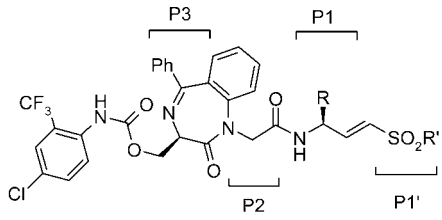
10.1021/jm701141u CCC: $40.75
Published on Web 01/31/2008

Chart 1. Structure of FP-2 Reversible Inhibitor **1**



1

Chart 2. Structure of the FP-2 Irreversible Inhibitors **2**–**3**



2a, R=CH_2CH_2Ph R'=CH_3
2b, R=CH_2CH_2Ph R'=C_2H_5
2c, R=CH_2CH_2Ph R'=Ph
2d, R=CH_2CH_2Ph R'=4-CH_3OPh

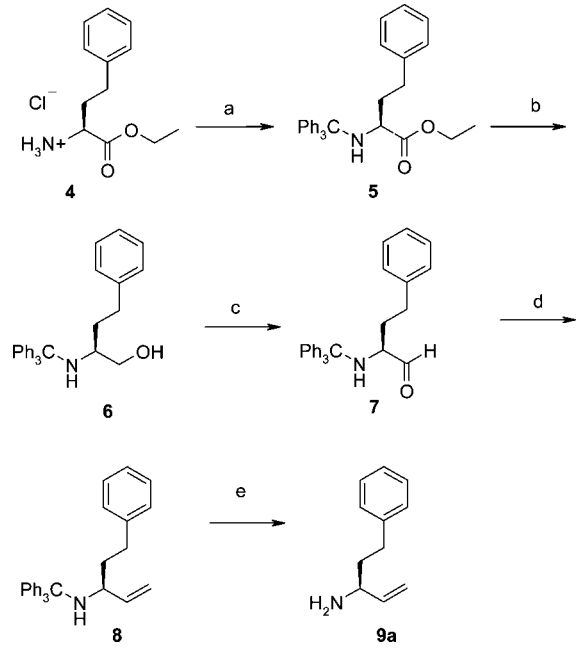
3a, R=H R'=CH_3
3b, R=H R'=C_2H_5
3c, R=H R'=Ph
3d, R=H R'=4-CH_3OPh

active of the series, is replaced by a vinyl sulfone moiety that spans P1 and P1′ sites. Vinyl sulfones are well-known covalent inhibitors of FP-2,[15] reported to inactivate the enzyme by irreversible addition of the thiol group of active site Cys-25 to the electrophilic vinyl sulfone moiety, which behaves as a Michael acceptor.[16] It is proposed that a hydrogen bond from the protonated active site His-159 to one of the sulfone oxygens might polarize the vinyl group, thus further activating the β-carbon toward nucleophilic attack (Figure 1).[17] The negative charge emerging at the α-carbon will immediately be eliminated via protonation by the histidinium residue.[18]

We focused our attention on the synthesis of two series of peptidomimetic vinyl sulfones, the first one containing at the P1 site the amino acid homoPhe (compounds **2a**−**d**, Chart 2), a residue known to boost the potency of FP-2 inhibitors.[15] The second series contains in the same position a glycine, useful to evaluate the relevance of the amino acid side chain in the process of recognition of the ligand by the enzyme. In both series, the vinyl sulfone moiety at the P1′ site bears various aromatic and aliphatic substituents chosen to evaluate the size and the characteristics of the P1′ lipophilic pocket, which could accommodate such a group.

With regard to the synthetic approach, we introduced the pharmacophore portion by olefin cross-metathesis,[19] a unique carbon skeleton redistribution in which unsaturated carbon−carbon bonds are rearranged in the presence of metal carbene complexes.[20] The advantages of this approach are that (i) high yields can be obtained under mild conditions in short reaction times, (ii) a wide range of functional groups are tolerated, with minimal substrate protection necessary, and (iii) the products are less expensive to prepare than those associated with other common catalytic C−C bond forming reactions.[21]

Scheme 1[a]



[a] Reagents and conditions: (a) Ph_3CCl, Et_3N, CH_2Cl_2, room temperature (rt), 12 h; (b) DIBALH, toluene, −78 °C, 3 h; (c) DMSO/oxalyl chloride, −78 °C, then TEA, −23 °C, 40 min; (d) $Ph_3PCH_3^+ I^-$, BuLi, THF, 0 °C−rt, 5 h; (e) HCl/acetone, reflux, 3 h.

All compounds were tested on recombinant FP-2[22] and on cultured *Plasmodia* in order to establish the structure–activity relationship. In order to check the selectivity toward papain-family human cysteine proteases, these compounds were tested against cathepsins B and L.

Results and Discussion

Chemistry. To synthesize the required P1 homoPhe derivative **9a** (Scheme 1), we started by protecting the α-amino group of (*S*)-2-amino-4-phenylbutyric acid ethyl ester (**4**)[23] with triphenylmethyl chloride in the presence of Et_3N. The resulting derivative **5** was then reduced with DIBALH to give alcohol **6**, which was in turn converted by Swern oxidation to the corresponding aldehyde **7** in high yield. Wittig reaction of the latter aldehyde **7** with triphenylmethyl phosphonium iodide in the presence of BuLi smoothly afforded olefin **8**. Finally, deprotection under acidic conditions gave the required (*S*)-1-phenethyl-allylamine (**9a**).

With amine **9a** in hand, we proceeded to its coupling with acid **10** (Scheme 2), a benzodiazepine derived mimic of the dipeptide fragment D-Ser-Gly, prepared according to a previously reported method.[14]

O-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) was employed, to assemble intermediate **11a** in high yield and on a preparative scale. Finally, fluoride promoted desilylation of **11a**, followed by treatment with 4-chloro-2-trifluoromethylphenyl isocyanate,

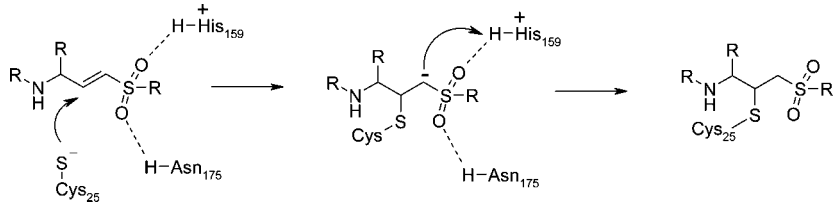
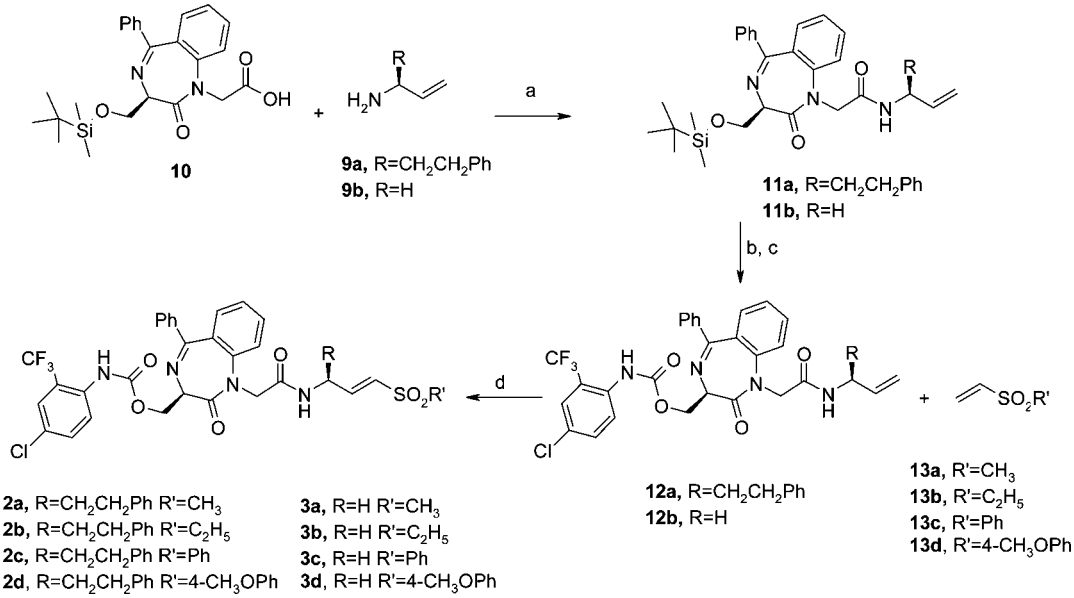


Figure 1. Mechanism of inhibition of FP-2 by vinyl sulfones. For the numbering of the residues, see ref 3.

Scheme 2[a]



[a] Reagents and conditions: (a) HATU, CH_2Cl_2, rt, 12 h; (b) TBAF, THF, rt, 5 h; (c) 4-Cl, 2-$CF_3C_6H_3NCO$, rt, 12 h; (d) Hoveyda catalyst, CH_2Cl_2, 100 °C, MW, 2 h.

afforded carbamate **12a**, a fully functionalized scaffold spanning the P1−P4 region and bearing the required terminal olefin handle in readiness for further functionalization *via* cross-metathesis methodology. In an analogous manner, tripeptide **12b**, containing Gly instead of homoPhe at P1, was obtained by coupling acid **10** with the commercially available allylamine **9b** (Scheme 2).

The introduction of the required vinyl sulfone warhead has been realized by cross-metathesis reaction using Hoveyda−Grubbs second generation catalyst,[24] a phosphine-free N-heterocyclic carbene ruthenium complex, which actually represents the catalyst of choice for these reactions. Thus, cross-metathesis reactions between the tripeptides **12a** or **12b** and vinyl sulfones **13a**−**13d** proceeded under microwave irradiation[25] in an efficient and rapid manner to afford compounds **2a**−**2d** and **3a**−**3d** as *E*-isomers.

Biological Activity. All compounds **2a**−**2d** and **3a**−**3d** were tested on recombinant FP-2 by using Cbz-Phe-Arg-AMC as fluorogenic substrate.[22] First, a preliminary screening with inhibitor concentrations of 50 μM was performed. An equivalent volume of DMSO was used as negative control, and the irreversible standard inhibitor of clan CA family C1 cysteine proteases (papain-family), namely E-64,[26] was used as positive control. The screening showed all compounds to abolish enzyme activity. Continuous assays (progress curve method)[27] (see Figures 2 and 3 for inhibition of FP-2 by compound **3a**) were then performed to determine the first-order rate constants of inhibition k_{inac} (min^{-1}), the dissociation constants K_{inac} (μM), and the second-order rate constants of inhibition k_{2nd} (M^{-1} min^{-1}), as $k_{2nd} = k_{inac}/K_{inac}$ (Table 1). The irreversible inhibition was confirmed by dialysis assays with compound **2c** (Figure 4).

As can be seen from the data reported in Table 1, all compounds exhibit high second-order rate constants, in the range of 161–634 × 10^3 M^{-1} min^{-1}. Although the range of the k_{2nd} values is not so extensive, a comparison of the k_{2nd} values of compounds **2a**−**2d** bearing at the P1 site the homoPhe side chain and those having a Gly residue in the same position (i.e., **3a**−**3d**) puts in evidence that the P1 substituent has a relevant impact on inhibitory kinetics. Compounds **2a**−**2d** showed higher second order rate constants with respect to analogues **3** (e.g., k_{2nd} = 432 × 10^3 M^{-1} min^{-1} for **2a** vs 161 × 10^3 M^{-1} min^{-1} for **3a**), with the exception of phenyl sulfone (**2c**) versus **3c**. The favorable influence of the homoPhe side chain is consistent

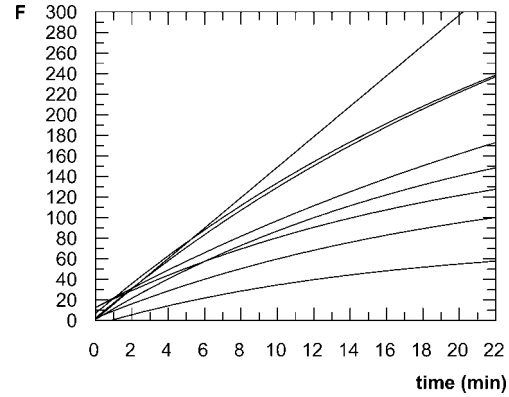


Figure 2. Progress curves of substrate hydrolysis in the presence of inhibitor **3a**. F = fluorescence units. Substrate: Cbz-Phe-Arg-AMC, 32 μM. Inhibitor concentrations (from top to bottom): 0, 0.5, 1, 2, 4, 6, 8, and 10 μM.

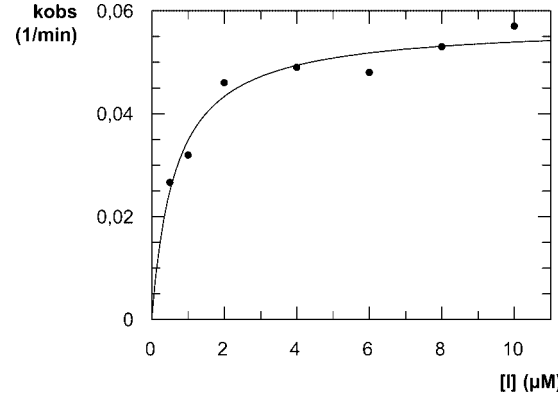


Figure 3. Plot of k_{obs} vs the inhibitor concentration for the inhibition of FP-2 by **3a**. k_{inac} = 0.059 (± 0.006) min^{-1}; $K_{inac} = K_{iapp}/(1 + [S]/K_m)$ = 0.37 (± 0.15) μM; $k_{2nd} = k_{inac}/K_{inac}$ = 161 ± 28 × 10^3 M^{-1} min^{-1}.

Table 1. Inhibition of FP-2 and Antiplasmodial Activity of Compounds **2**−**3**

compound	k_{2nd} ($\times 10^3$ M^{-1} min^{-1})	k_{inac} (min^{-1})/K_{inac} (μM)	*P. falciparum* IC_{50} (μM)
2a	432 ± 63	0.16 (±0.03)/0.39 (±0.08)	55.4
2b	307 ± 12	0.10 (±0.05)/0.32 (±0.08)	9.1
2c	175 ± 15	0.11 (±0.01)/0.60 (±0.4)	9.2
2d	634 ± 60	0.034 (±0.008)/0.053 (±0.012)	59.6
3a	161 ± 28	0.059 (±0.006)/0.37 (±0.15)	18.1
3b	248 ± 22	0.027 (±0.002)/0.11 (±0.04)	56.6
3c	243 ± 7	0.033 (±0.004)/0.14 (±0.01)	23.1
3d	291 ± 33	0.031 (±0.01)/0.12 (±0.04)	>100
E-64	1700 ± 400[a]	0.46 (±0.07)/0.29 (±0.09)	5.3[b]

[a] $k_{ass} = k_{inac}/K_{iapp} = 11.31$ (±2.70) $\times 10^3$ M^{-1} s^{-1}; literature value[26] with 25 μM Cbz-Leu-Arg-AMC as substrate: $k_{ass} = 12.28$ (±1.40) $\times 10^3$ M^{-1} s^{-1}. All results include standard deviations from two independent measurements, each performed in duplicate. [b] Literature value.[28]

Table 2. Inhibition of Cathepsins B and L of Compounds **2**−**3**

	cathepsin B		cathepsin L	
compound	k_{2nd} ($\times 10^3$ M^{-1} min^{-1})	k_{inac} (min^{-1})/K_i (μM)	k_{2nd} ($\times 10^3$ M^{-1} min^{-1})	k_{inac} (min^{-1})/K_i (μM)
2a	4.4 ± 1.2	0.064 (±0.003)/14.5 (±2.7)	3.5 ± 2.0	[a]
2b	10.3 ± 1.4	0.045 (±0.003)/4.4 (±2.1)	7.3 ± 1.0	0.10 (±0.02)/13.5(±1.3)
2c	11.5 ± 1.3	0.039 (±0.003)/3.1 (±2.5)	16.5 ± 3.9	0.12 (±0.01)/7.0(±2.9)
2d	13.7 ± 2.1	0.045 (±0.003)/3.3 (±1.5)	1.0 ± 0.05	[a]
3a	6.6 ± 1.1	0.048 (±0.004)/11.0 (±3.4)	0.88 ± 0.04	[a]
3b	11.7 ± 5.4	0.039 (±0.002)/4.3 (±2.1)	1.0 ± 0.09	[a]
3c	12.6 ± 5.3	0.047 (±0.001)/4.6 (±2.0)	7.5 ± 0.8	0.16 (±0.03)/21.9(±6.0)
3d	10.0 ± 1.0	0.059 (±0.009)/5.9 (±0.4)	7.6 ± 1.3	0.10 (±0.03/14.0(±6.8)

[a] Since the k_{obs} vs [I] diagrams were restricted to the linear range, only the second-order rate constant could be determined as k_{obs}/[I]. All of the results include standard deviations from two independent measurements, each performed in duplicate.

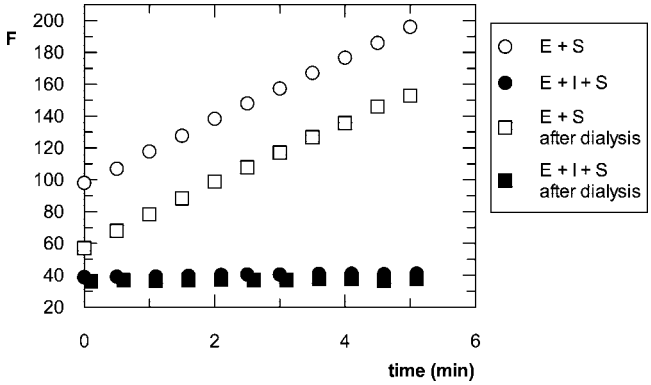


Figure 4. Progress curves for the inhibition of FP-2 by **2c**. The dialysis of the incubation mixture of enzyme E (13 μg mL^{-1}) and inhibitor I (100 μM) with a 5000-fold excess of buffer prior to addition of substrate S (32 μM) did not lead to the regeneration of enzyme activity, proving the irreversible inhibition. F = fluorescence units.

with previously reported studies on peptidyl vinyl sulfones.[15] Furthermore, although there is not a clear correlation between the nature of the substituent on the sulfone portion and inhibitory properties, an inhibition improvement is reached with the introduction of a methoxy substituent at the 4′ position of the P1′ phenyl moiety that as a hydrogen bond acceptor could steady the enzyme–inhibitor interaction.

In terms of inhibition rate, the introduction of the homoPhe at the P1 site led to compounds (**2a**−**2c**) that showed values of first-order rate constant of inactivation higher than those of Gly derivatives (**3a**−**3c**), whereas the low k_{inac} value for derivative **2d** should be due to the presence of the electron donating methoxy group. It is noteworthy that the presence of this group in compound **2d** has a favorable effect on the affinity for the enzyme, leading to a 10-fold improvement of K_{inac} (K_{inac} = 53 nM for **2d** vs K_{inac} = 0.6 μM for **2c**).

Selectivity assays were also performed, testing inhibitors against papain-family human cysteine proteases such as cathepsins B and L. The results of the evaluation indicate that all compounds **2** and **3** are weak inhibitors of cathepsin B and particularly of cathepsin L (Table 2). The second-order rate constants for cathepsins B and L inhibition are 1–2 orders of magnitude lower than those for FP-2 inhibition, so demonstrating that all new vinyl sulfones are quite selective toward FP-2.

For all compounds an additional assay with the *P. falciparum* strain 3D7 was performed. Dose-dependent effects of compounds on parasite development (*P. falciparum* strain 3D7) were quantified using the Malstat assay, which measures the activity of the *Plasmodium*-specific enzyme lactate dehydrogenase as described previously[29] (Table 1). Although the antiplasmodial activity is not directly related with the inhibitory potency, a comparison of the results of derivatives **2** and **3** confirmed that the presence of the homoPhe residue at the P1 site is preferable to the Gly one (e.g., IC_{50} = 9.1 μM for **2b** vs IC_{50} = 56.6 μM for **3b**), with the only exception of **3a** versus **2a**. It is noteworthy that compound **2c**, one of the less potent inhibitors, displayed a high antiplasmodial activity (i.e., IC_{50} 9.2 μM). However, the presence of the methoxy group at the 4′ position of the P1′ phenyl ring, which strongly steadies the in vitro enzyme–inhibitor interaction (i.e., **2c** versus **2d**), did not induce a parallel increase of the antiparasitic activity. A possible explanation of the poor activity of the most potent inhibitor **2d** against cultured parasites could be a limited access to the intracellular protease target.

In conclusion, we designed and synthesized new peptidomimetics **2**–**3**, which proved to be potent and selective, irreversible inhibitors of FP-2 with a significant antiplasmodial activity. Compounds **2b** and **2c**, which possess a good inhibitory potency coupled with the highest activity against cultured *Plasmodium*, represent lead compounds for further development in the design of new inhibitors which can be used as antimalarial agents.

Experimental Section

All reagents and solvents were obtained from commercial suppliers and were used without further purification. With the exception of routine deprotection steps, reactions were performed in oven-dried glassware under an atmosphere of nitrogen. Reactions under microwave irradiation were performed on a Biotage Emrys Optimizer apparatus, with the same software protocol reported in

ref 25. Elemental analyses were carried out on a C. Erba Model 1106 elemental analyzer for C, H, and N, and the results are within ±0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC. Flash chromatography purifications were performed on Merck silica gel (200–400 mesh) as stationary phase or were conducted using prepacked cartridges on a Biotage system. Preparative HPLC was performed on the following: (1) An automated (mass-triggered) RP-HPLC Waters Micromass system, incorporating a 2525 pump module, a Micromass ZMD detector, and a 2767 collection module and operating under Fraction Lynx software. The mobile phase comprised a linear gradient of a binary mixture of MeCN (containing 0.1% TFA) and water (containing 0.1% TFA). The flow rate was 20 mL/min, and the stationary phase was a Simmetry C_{18} column (7 μm, 19 mm × 300 mm). Gradient A: 50% MeCN for 2 min; from 50% MeCN to 90% MeCN in 14 min. Gradient B: 40% MeCN for 2 min; from 40% MeCN to 90% MeCN in 14 min. (2) A Waters 996 system equipped with a photodiode array detector. The solvent system was MeCN−0.1% TFA/water−0.1% TFA with a flow rate of 15 mL/min using an XTerra C_{18} column (5 μm, 19 mm × 100 mm). Gradient C: 15% MeCN for 3 min; from 15% MeCN to 90% MeCN in 15 min. RP-HPLC-MS analyses were performed on a Water Alliance 2795 apparatus, equipped with a diode array detector and a ZQ mass spectrometer, using an XTerra C_{18} column (5 μm, 4.6 mm × 50 mm). Flow rate: 1 mL/min. The solvent system was MeCN−0.1% HCOOH/water−0.1% HCOOH. Gradient D: from 10% MeCN to 50% MeCN in 0.5 min; from 50% MeCN to 100% MeCN in 6 min. Gradient E: from 10% MeCN to 100% MeCN in 7.5 min. RP-UPLC-MS analyses were performed on a Waters Acquity apparatus, equipped with a PDA and a Micromass ZQ mass spectrometer, using an Acquity C_{18} column (1.7 μm, 2.1 mm × 50 mm). Flow rate: 0.5 mL/min. The solvent system was MeCN−0.1% HCOOH/water−0.1% HCOOH. Gradient F: From 10% MeCN to 100% MeCN in 0.50 min, then 3 min at 100% MeCN. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AM series spectrometer and unless otherwise stated were recorded at 300 K and 300 and 75 MHz, respectively. ^{1}H chemical shifts are reported in ppm downfield from internal TMS. ^{13}C chemical shifts are referenced to $CDCl_3$ (central peak, $\delta = 77.0$ ppm). Compound **10** was synthesized in a 72% overall yield optimizing an already reported procedure.[14] Details of the procedures are reported in the Supporting Information.

(*S*)-4-Phenyl-2-(tritylamino)-butyric Acid Ethyl Ester (5). A mixture of (*S*)-2-amino-4-phenylbutyric acid ethyl ester hydrochloride (**4**) (5 g, 20 mmol), triphenylmethyl chloride (6.1 g, 22 mmol), and Et_3N (6.13 mL, 44 mmol) in dry CH_2Cl_2 (200 mL) was stirred at room temperature for 12 h. After this time, the mixture was washed with citric acid and water. The organic phase was dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography using as eluent petroleum ether/EtOAc (95/5) to afford title compound **5** (7.9 g, 89%). RP-HPLC-MS: gradient D, retention time 5.95 min. MS (ESI$^+$) *m*/*z* 450.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.04 (t, $J = 7.1$ Hz, 3H), 2.01–2.17 (m, 2H), 2.55–2.79 (m, 2H), 3.35–3.55 (m, 2H), 3.63 (m, 1H), 7.14–7.29 (m, 15H), 7.49 (d, $J = 8.0$ Hz, 5H). ^{13}C NMR: 14.15, 30.64, 33.58, 55.79, 61.33, 67.56, 126.18, 126.34, 128.34, 128.93, 129.38, 138.13, 145.01, 171.66.

(*S*)-4-Phenyl-2-(tritylamino)-butan-1-ol (6). To a solution of (*S*)-4-phenyl-2-(tritylamino)-butyric acid ethyl ester (**5**) (7.9 g, 17.8 mmol) in dry toluene (90 mL) at −78 °C was added DIBALH (25.6 g, 178 mmol). After 2 h at −78 °C, the mixture was quenched with MeOH (20 mL) followed by addition of a saturated solution of Rochelle salt (200 mL). The mixture was allowed to warm to 25 °C, and diethyl ether (100 mL) was added. The organic layer was dried (Na_2SO_4), and the solvent was removed under reduced pressure. The title compound **6** (7.2 g, 99%) was used directly in the following step. RP-HPLC-MS: gradient D, retention time 3.14 min. MS (ESI$^+$) *m*/*z* 408.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.43–1.63 (m, 2H), 1.85 (br s, 1H), 2.21–2.49 (m, 2H), 2.67 (m, 1H), 3.15 (dd, $J = 10.8$, 4.2 Hz, 1H), 3.32 (dd, $J = 10.8$, 2.9 Hz, 1H), 7.12 (d, $J = 6.9$ Hz, 2H), 7.36–7.53 (m, 13H), 7.52 (d, $J = 7.5$ Hz, 5H).^{13}C NMR: 31.25, 33.19, 51.92, 67.30, 68.23, 126.09, 126.35, 128.25, 128.36, 128.62, 129.36, 138.73, 145.01.

(*S*)-4-Phenyl-2-(tritylamino)-butyraldehyde (7). To a 2 M solution of oxalyl chloride in CH_2Cl_2 (15 mL, 30 mmol) at −65 °C was added a solution of DMSO (5 mL, 71.2 mmol) in CH_2Cl_2 (6 mL) dropwise over a period of 10 min. A solution of alcohol **6** (7.2 g, 17.8 mmol) in CH_2Cl_2 (10 mL) was added dropwise over 20 min. The reaction mixture was stirred at −65 °C for 45 min, then Et_3N (16 mL, 115.7 mmol) was added dropwise over a period of 10 min, and the mixture was stirred at −23 °C for a further 40 min. The solvent was then evaporated under reduced pressure, and the residue was taken up into diethyl ether (200 mL) and 0.5 N $KHSO_4$ solution (200 mL). The organic layer was separated, washed with water (200 mL) and brine (200 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The crude aldehyde **7** (7.2 g, 99%) was used for the next step without any further purification. RP-HPLC-MS: gradient D, retention time 5.20 min. MS (ESI$^+$) *m*/*z* 406.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.73–1.82 (m, 2H), 2.50 (m, 1H), 2.71 (m, 1H), 3.43 (m, 1H), 7.09–7.31 (m, 15H), 7.45–7.58 (m, 5H), 9.01 (s, 1H). ^{13}C NMR: 30.62, 30.73, 66.06, 67.63, 126.18, 126.34, 128.25, 128.32, 128.99, 129.32, 138.15, 145.02, 200.20.

(*S*)-(1-Phenethylallyl)-tritylamine (8). To a suspension of triphenylphosphonium iodide (14.39 g, 35.6 mmol) in dry THF (360 mL) at 0 °C was added BuLi (1.6 M solution in THF, 22.2 mL, 35 mmol) dropwise over 10 min. The resulting clear orange solution was stirred at 0 °C for 10 min before adding aldehyde **7** (7.20 g, 17.8 mmol) as a solution in dry THF (20 mL). The resulting mixture was allowed to warm to room temperature over a period of 3 h, and then hexane (200 mL) was added. The resulting precipitate was filtered off, and the filtrate was washed with water (2 × 200 mL). The organic phase was separated, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by flash chromatography eluting with petroleum ether/EtOAc (1/1) to give compound **8** (6.45 g, 90%) as a yellow oil. RP-HPLC-MS: gradient D, retention time 5.75 min. MS (ESI$^+$) *m*/*z* 404.0 [M + H]$^+$ (100%). ^{1}H NMR: 0.83–0.90 (m, 2H), 2.23 (m, 1H), 2.41 (m, 1H), 3.04 (q, $J = 6.6$ Hz, 1H), 4.88–4.94 (m, 2H), 5.61 (m, 1H), 6.91 (d, $J = 7.1$ Hz, 2H), 7.09–7.32 (m, 12H), 7.52 (d, $J = 7.1$ Hz, 6H). ^{13}C NMR: 31.32, 38.13, 51.96, 68.24, 115.87, 126.18, 126.34, 128.22, 128.38, 128.94, 129.35, 135.21, 138.11, 145.06.

(*S*)-1-Phenethylallylamine (9a). To a solution (*S*)-(1-phenethylallyl)-tritylamine **8** (6.45 g, 16 mmol) in acetone (80 mL) was added 6 N HCl (4 mL, 24 mmol) dropwise over 5 min, and the resulting mixture was refluxed for 5 h. The solvent was then evaporated under reduced pressure, the residue was taken up in CH_2Cl_2 (200 mL), and the solution was extracted with 1 N HCl (2 × 200 mL). The aqueous phase was brought to pH = 10 by addition of solid Na_2CO_3 and extracted with CH_2Cl_2 (2 × 200 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated under reduced pressure to give amine **9a** (2.26 g, 88%). RP-HPLC-MS: gradient D, retention time 1.53 min. MS (ESI$^+$) *m*/*z* 162.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.92–1.99 (m, 2H), 2.57–2.70 (m, 2H), 3.94 (q, $J = 6.5$ Hz, 1H), 5.01–5.07 (m, 2H), 5.79 (m, 1H), 7.15–7.21 (m, 3H), 7.25–7.29 (m, 2H). ^{13}C NMR: 31.01, 39.75, 54.04, 115.87, 126.15, 128.27, 128.93, 135.27, 138.15.

2-[(3*R*)-3-({[*tert*-Butyl(dimethyl)silyl]oxy}methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-1-yl]-*N*-[(1*S*)-1-(2-phenylethyl)prop-2-en-1-yl]acetamide (11a). To a solution of [(3*R*)-3-({[*tert*-butyl(dimethyl)silyl]oxy}methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-1-yl]acetic acid (**10**) (2.2 g, 5 mmol) in CH_2Cl_2 (50 mL) at 0 °C was added HATU (2.85 g, 7.5 mmol). After 5 min, the ice bath was removed, and a solution of amine **9a** (1.62 g, 10 mmol) in CH_2Cl_2 (5 mL) was added. The resulting mixture was stirred at room temperature for 12 h and washed with water (2 × 50 mL). The organic layer was separated, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography, eluting with petroleum ether/EtOAc (8/2) to give title compound **11a** (2.6 g, 90%) as a yellow oil. RP-HPLC-MS: gradient D, retention time 5.72 min. MS (ESI$^+$) *m*/*z* 582.1 [M + H]$^+$ (100%). ^{1}H NMR: 0.17

(s, 3H), 0.19 (s, 3H), 0.99 (s, 9H), 1.62–1.75 (m, 2H), 2.58 (t, J = 8.0 Hz, 2H), 3.73 (t, J = 6.4 Hz, 1H), 4.21 (d, J = 15.2 Hz, 1H), 4.25 (m, 1H), 4.38–4.50 (m, 2H), 4.55 (d, J = 15.2 Hz, 1H), 4.49–5.19 (m, 2H), 5.68 (m, 1H), 7.10–7.70 (m, 14H). ^{13}C NMR: −5.69, 18.07, 26.02, 31.06, 37.06, 50.87, 54.37, 63.01, 68.43, 115.80, 121.47, 125.01, 126.15, 127.63, 128.72, 128.89, 129.52, 129.54, 131.21, 131.53, 135.28, 138.18, 139.10, 140.87, 170.89, 171.12.

***N*-Allyl-2-[(3*R*)-3-({[*tert*-butyl(dimethyl)silyl]oxy}methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-1-yl]acetamide (11b).** Compound **11b** was obtained from **10** (2.2 g, 5 mmol) and amine **9b** (571 mg, 0.75 mL, 10 mmol) employing the procedure described for compound **11a**. Compound **11b** (2.36 g, 99%) was used without further purification in the following step. RP-HPLC-MS: gradient D, retention time 4.67 min. MS (ESI$^+$) m/z 478.1 [M + H]$^+$ (100%). ^{1}H NMR: 0.17 (s, 3H), 0.19 (s, 3H), 0.99 (s, 9H), 3.74 (t, J = 6.6 Hz, 1H), 3.80 (d, J = 6.4 Hz, 2H), 4.14–4.32 (m, 2H), 4.20 (d, J = 15.3 Hz, 1H), 4.50 (d, J = 15.3 Hz, 1H), 4.93–5.02 (m, 2H), 5.67 (m, 1H), 6.30 (bt, 1H), 7.14–7.60 (m, 9H). ^{13}C NMR: −5.75, 18.16, 25.84, 41.76, 54.06, 62.97, 68.19, 116.24, 121.77, 124.59, 127.32, 128.93, 129.25, 129.46, 131.17, 131.34, 134.38, 139.09, 140.74, 171.17, 171.21.

[(3*R*)-2-Oxo-1-(2-oxo-2-{[(1*S*)-1-(2-phenylethyl)prop-2-en-1-yl]amino}ethyl)-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]methyl[4-chloro-2-(trifluoromethyl)phenyl]carbamate (12a). Step 1. To a solution of **11a** (2.61 g, 4.5 mmol) in dry THF (45 mL) was added tetrabutylammonium fluoride (TBAF) (1 M solution in THF, 6.75 mL, 6.75 mmol) dropwise. The mixture was stirred at room temperature until disappearance of the starting material (TLC monitoring), and then it was diluted with EtOAc (100 mL) and washed with water (2 × 100 mL). The organic layer was separated, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to give the deprotected compound (2.05 g, 99%) as a yellow oil, which was used in the next step without further purification. RP-HPLC-MS: gradient D, retention time 2.66 min. MS (ESI$^+$) m/z 468.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.71–1.76 (m, 2H), 2.55 (t, J = 7.9 Hz, 2H), 3.87 (t, J = 6.3 Hz, 1H), 4.21 (m, 1H), 4.27 (d, J = 15.3 Hz, 1H), 4.36–4.48 (m, 2H), 4.57 (d, J = 15.3 Hz, 1H), 5.07–5.15 (m, 2H), 5.71 (m, 1H), 6.20 (d, J = 8.2 Hz, 1H), 7.07–7.72 (m, 14H). Step 2. To a solution of 2-[(3*R*)-3-(hydroxymethyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-1-yl]-*N*-[(1*S*)-1-(2-phenylethyl)prop-2-en-1-yl]acetamide (2.05 g, 4.4 mmol) in dry CH_2Cl_2 (45 mL) was added 4-Cl,2-$CF_3C_6H_3NCO$ (1.94 g, 1.32 mL, 8.8 mmol), and the resulting mixture was stirred for 12 h at room temperature. After this time, PS-Trisamine (5 equiv) was added and the mixture was stirred for a further 1 h, then filtered, and washed with water (2 × 50 mL). The residue was purified by flash chromatography, eluting with petroleum ether/EtOAc (7/3), to afford compound **12a** (2.27 g, 75%). RP-HPLC-MS: gradient D, retention time 4.70 min. MS (ESI$^+$) m/z 689.1 [M + H]$^+$ (100%). ^{1}H NMR: 1.63–1.75 (m, 2H), 2.49 (t, J = 8.0 Hz, 2H), 3.79 (t, J = 6.4 Hz, 1H), 4.11 (m, 1H), 4.15 (d, J = 15.2 Hz, 1H), 4.29–4.44 (m, 2H), 4.48 (d, J = 15.2 Hz, 1H), 4.49–5.12 (m, 2H), 5.66 (m, 1H), 6.10 (d, J = 8.2 Hz, 1H), 7.01–7.69 (m, 17H). ^{13}C NMR: 31.05, 37.03, 50.86, 54.38, 60.35, 65.88, 114.65, 115.83, 121.71, 121.94, 123.35, 124.56, 125.28, 126.19, 127.32, 128.21, 128.94, 129.06, 129.27, 129.45, 130.27, 131.18, 131.35, 132.48, 135.29, 138.14, 139.07, 140.73, 153.92, 168.67, 170.88, 171.29.

{(3*R*)-1-[2-(Allylamino)-2-oxoethyl]-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl}methyl [4-Chloro-2-(trifluoromethyl)phenyl]carbamate (12b). Compound **12b** was obtained from **11b** (2.36 g, 4.9 mmol) by employing the same procedure described for compound **12a**. Intermediate *N*-allyl-2-[(3*R*)-3-(hydroxymethyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-1-yl]acetamide was obtained in 99% yield (1.74 g). RP-HPLC-MS: gradient D, retention time 1.76 min. MS (ESI$^+$) m/z 364.1 [M + H]$^+$ (100%). ^{1}H NMR: 3.75 (t, J = 6.6 Hz, 1H), 3.81 (d, J = 6.4 Hz, 2H), 4.16–4.34 (m, 2H), 4.25 (d, J = 15.3 Hz, 1H), 4.54 (d, J = 15.3 Hz, 1H), 4.94–5.03 (m, 2H), 5.69 (m, 1H), 6.32 (bt, 1H), 7.16–7.61 (m, 9H). Title compound **12b** was purified by flash chromatography, eluting with petroleum ether/EtOAc (5/5) (2.33 g, 80%). RP-HPLC-MS: gradient D, retention time 3.60 min. MS (ESI$^+$) m/z 585.0 [M + H]$^+$ (100%). ^{1}H NMR: 3.76 (t, J = 6.4 Hz, 2H), 3.98 (t, J = 6.6 Hz, 1H), 4.28 (d, J = 15.3 Hz, 1 H), 4.58 (d, J = 15.3 Hz, 1 H), 4.95 (dd, J = 11.2, 6.3 Hz, 1H), 5.03–5.12 (m, 3H), 5.72 (m, 1H), 6.22 (bt, 1H), 6.91 (s, 1H), 7.23–7.36 (m, 4H), 7.39–7.44 (m, 2H), 4.47 (d, J = 2.2 Hz, 1H), 7.56 (m, 3H), 7.97 (d, J = 8.6 Hz, 1H), 8.04 (d, J = 8.6 Hz, 1H). ^{13}C NMR: 41.75, 54.03, 60.38, 65.86, 114.62, 116.25, 121.75, 121.98, 123.35, 124.58, 125.25, 127.34, 128.97, 129.08, 129.24, 129.46, 130.27, 131.17, 131.32, 132.45, 134.38, 139.04, 140.75, 153.97, 168.67, 171.12, 171.24.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-[(3-Methanesulfonyl-(1*S*)-1-phenethyl-allylcarbamoyl)-methyl]-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (2a). To a solution of **12a** (50 mg, 0.072 mmol) in dry CH_2Cl_2 (5 mL) was added methyl vinyl sulfone (**13a**) (77 mg, 0.72 mmol) followed by Hoveyda−Grubbs second generation catalyst [(1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene)-dichloro-(*O*-isopropoxyphenylmethylene)ruthenium] (4.5 mg, 0.0072 mmol). The resulting mixture was heated under microwave irradiation at 100 °C for 2 h. The solvent was then removed under reduced pressure, and the residue was purified by preparative RP-HPLC (Waters 996 system, gradient C) to give the title compound as a solid (28.9 mg, 47.2%). RP-UPLC-MS: gradient F, retention time 2.29 min. MS (ESI$^+$) m/z 767.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.21–1.39 (m, 2H), 2.56–2.63 (m, 2H), 2.92 (s, 3H), 4.03 (t, J = 6.0 Hz, 1H), 4.30 (d, J = 15.3 Hz, 1H), 4.48 (d, J = 15.3 Hz, 1H), 4.66 (m, 1H), 4.90–5.06 (m, 2H), 6.30 (d, J = 8.6 Hz, 1H), 6.59 (d, J = 15.2 Hz, 1H), 6.81 (dd, J = 15.2, 4.2 Hz, 1H), 7.08–7.66 (m, 15H), 7.75 (d, J = 9.3 Hz, 1H), 8.2 (d, J = 9.1 Hz, 1H). ^{13}C NMR: 30.98, 36.89, 43.25, 48.98, 54.23, 60.45, 65.78, 114.86, 121.67, 121.79, 123.83, 124.85, 125.32, 126.21, 127.43, 128.52, 128.69, 129.70, 129.72, 129.84, 129.95, 130.12, 131.21, 131.33, 132.44, 138.21, 138.30, 139.10, 140.67, 154.19, 168.36, 170.68, 171.02. Anal. ($C_{38}H_{34}ClF_3N_4O_6S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-[(3-Ethanesulfonyl-(1*S*)-1-phenethyl-allylcarbamoyl)-methyl]-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (2b). Compound **12a**(50 mg, 0.072 mmol) was reacted with ethyl vinyl sulfone (**13b**) (88 mg, 0.72 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters Micromass system, gradient A) as a solid (30.9 mg, 49.5%). RP-UPLC-MS: gradient F, retention time 2.33 min. MS (ESI$^+$) m/z 781.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.30 (t, J = 7.5 Hz, 3H), 1.85–2.04 (m, 2H), 2.58–2.63 (m, 2H), 2.97 (q, J = 7.5 Hz, 2H), 4.03 (t, J = 6.7 Hz, 1H), 4.27 (d, J = 15.0 Hz, 1H), 4.49 (d, J = 15.0 Hz, 1H), 4.66 (m, 1H), 4.91–5.05 (m, 2H), 6.32 (d, J = 8.4 Hz, 1H), 6.49 (d, J = 15.9 Hz, 1H), 6.79 (dd, J = 15.9, 4.3 Hz, 1H), 7.08–7.62 (m, 15H), 7.74 (d, J = 8.0 Hz, 1H), 7.9 (d, J = 9.1 Hz, 1H). ^{13}C NMR: 5.15, 31.04, 37.01, 45.48, 49.25, 54.26, 60.36, 65.88, 114.67, 121.79, 121.98, 123.35, 124.52, 125.27, 126.18, 127.39, 128.21, 128.92, 129.07, 129.29, 129.46, 129.53, 130.29, 131.14, 131.78, 132.46, 138.08, 138.15, 139.08, 140.73, 153.99, 168.63, 170.89, 171.24. Anal. ($C_{39}H_{36}ClF_3N_4O_6S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-{[3-Benzenesulfonyl-(1*S*)-1-phenethyl-allylcarbamoyl]-methyl}-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (2c). Compound **12a** (50 mg, 0.072 mmol) was reacted with vinyl phenyl sulfone (**13c**) (121 mg, 0.72 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters Micromass system, gradient A) as a solid (32 mg, 48.2%). RP-UPLC-MS: gradient F, retention time 2.10 min. MS (ESI$^+$) m/z 829.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.68–1.80 (m, 2H), 2.54 (t, J = 8.0 Hz, 2H), 4.06 (t, J = 6.4 Hz, 1H), 4.30 (d, J = 15.2 Hz, 1H), 4.46 (m, 1H), 4.60 (d, J = 15.2 Hz, 1H), 4.89–5.01 (m, 2H), 6.52 (d, J = 15.3 Hz, 1H), 6.82 (dd, J = 15.3, 4.4 Hz, 1H),

7.03–7.96 (m, 21H), 8.06 (d, J = 9.1 Hz, 1H). ^{13}C NMR: 31.10, 37.23, 49.32, 54.23, 60.67, 65.78, 114.53, 121.45, 121.63, 123.23, 124.85, 125.32, 126.61, 127.23, 128.62, 128.83, 128.89, 129.30, 129.42, 129.64, 129.78, 129.98, 130.12, 131.41, 131.83, 132.34, 133.88, 138.11, 138.90, 139.34, 139.60, 139.86, 140.67, 154.12, 168.43, 170.18, 171.12. Anal. ($C_{43}H_{36}ClF_3N_4O_6S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-{[3-(4-Methoxy-benzenesulfonyl)-(1*S*)-1-phenethyl-allylcarbamoyl]-methyl}-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]-diazepin-3-ylmethyl Ester (2d). Compound **12a** (50 mg, 0.072 mmol) was reacted with 4-methoxyphenyl vinyl sulfone (**13d**) (144 mg, 0.72 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters Micromass system, gradient B) as a solid (31.8 mg, 46.4%). RP-UPLC-MS: gradient F, retention time 2.10 min. MS (ESI$^+$) m/z 858.9 [M + H]$^+$ (100%). ^{1}H NMR: 1.68–1.81 (m, 2H), 2.54 (t, J = 8.1, 2H), 3.81 (s, 3H), 4.06 (t, J = 6.4 Hz, 1H), 4.28 (d, J = 15.1 Hz, 1H), 4.46 (m, 1H), 4.59 (d, J = 15.1 Hz, 1H), 4.88–5.02 (m, 2H), 6.50 (d, J = 15.1 Hz, 1H), 6.78 (dd, J = 15.1, 4.2 Hz, 1H), 6.92 (d, J = 8.8 Hz, 2H), 7.06 (d, J = 9.7 Hz, 2H), 7.17–7.75 (m, 14H), 7.76 (d, J = 8.8, 2H), 8.09 (d, J = 9.1 Hz, 1H). ^{13}C NMR: 32.01, 37.10, 49.13, 53.93, 55.89, 60.39, 65.67, 113.96, 115.23, 121.57, 121.89, 123.35, 124.78, 125.45, 126.34, 127.46, 128.34, 128.89, 129.12, 129.44, 129.34, 129.56, 130.34, 131.31, 131.43, 131.70, 132.46, 138.10, 138.23, 139.23, 139.78, 140.67, 153.79, 165.75, 168.23, 170.48, 171.02. Anal. ($C_{44}H_{38}ClF_3N_4O_7S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-[(3-Methanesulfonyl-allylcarbamoyl)-methyl]-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (3a). Compound **12b** (50 mg, 0.085 mmol) was reacted with methyl vinyl sulfone (**13a**) (90 mg, 0.85 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters 996 system, gradient C) as a solid (25 mg, 47.5%). RP-UPLC-MS: gradient F, retention time 1.98 min. MS (ESI$^+$) m/z 663.0 [M + H]$^+$ (100%). ^{1}H NMR: 2.89 (s, 3H), 4.05–4.08 (m, 3H), 4.43 (d, J = 15.3 Hz, 1H), 4.61 (d, J = 15.3 Hz, 1H), 4.94 (m, 1H), 5.05 (m, 1H), 6.48 (d, J = 15.5 Hz, 1H), 6.65 (bt, 1H), 6.83 (m, 1H), 7.12 (bs, 1H), 7.33–7.69 (m, 11H), 7.99 (d, J = 9.3 Hz, 1H). ^{13}C NMR: 40.11, 43.25, 54.05, 60.30, 65.85, 114.63, 121.70, 121.94, 123.33, 124.52, 125.24, 127.32, 128.97, 129.05, 129.23, 129.44, 129.92, 130.21, 131.18, 131.35, 132.41, 134.34, 139.05, 140.74, 153.96, 168.63, 171.18, 171.26. Anal. ($C_{30}H_{26}ClF_3N_4O_6S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-[(3-Ethanesulfonyl-allylcarbamoyl)-methyl]-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (3b). Compound **12b** (50 mg, 0.085 mmol) was reacted with ethyl vinyl sulfone (**13b**) (102 mg, 0.85 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters Micromass system, gradient B) as a solid (28 mg, 53%). RP-UPLC-MS: gradient F, retention time 2.04 min. MS (ESI$^+$) m/z 677.0 [M + H]$^+$ (100%). ^{1}H NMR: 1.28 (t, J = 7.5 Hz, 3H), 2.93 (q, J = 7.5 Hz, 2H), 4.02–4.10 (m, 2H), 4.16 (t, J = 6.6 Hz, 1H), 4.45 (d, J = 15.5 Hz, 1H), 4.55 (d, J = 15.5 Hz, 1H), 4.92–5.04 (m, 2H), 6.39 (d, J = 15.1 Hz, 1H), 6.80 (m, 1H), 6.94 (bt, 1H), 7.18 (bs, 1H), 7.33–7.73 (m, 11H), 7.97 (d, J = 9.1 Hz, 1H). ^{13}C NMR: 5.11, 40.18, 45.48, 54.35, 60.28, 66.85, 115.63, 121.60, 122.94, 123.45, 124.82, 125.14, 128.32, 128.97, 129.44, 129.75, 129.87, 129.96, 130.81, 131.18, 131.35, 133.41, 135.34, 140.05, 140.43, 154.94, 169.33, 169.18, 171.23. Anal. ($C_{31}H_{28}ClF_3N_4O_6S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-[(3-Benzenesulfonyl-allylcarbamoyl)-methyl]-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (3c). Compound **12b** (50 mg, 0.085 mmol) was reacted with vinyl phenyl sulfone (**13c**) (143 mg, 0.85 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters Micromass system, gradient A) as a solid (28.4 mg, 49.2%). RP-UPLC-MS: gradient F, retention time 2.19 min. MS (ESI$^+$) m/z 746.9 [M + H]$^+$ (100%). ^{1}H NMR: 3.96–4.06 (m, 3H), 4.33 (d, J = 15.3 Hz, 1H), 4.59 (d, J = 15.3 Hz, 1H), 4.89–5.02 (m, 2H), 6.45 (d, J = 15.0 Hz, 1H), 6.59 (bt, 1H), 6.87 (m, 1H), 7.13 (bs, 1H), 7.28–7.68 (m, 14H), 7.86 (d, J = 7.3 Hz, 2H), 7.99 (d, J = 8.8 Hz, 1H). ^{13}C NMR: 38.35, 53.52, 60.75, 65.44, 122.67, 124.95, 125.02, 125.34, 126.98, 128.34, 128.76, 128.98, 129.32, 129.70, 129.86, 130.78, 130.90, 131.13, 132.23, 132.67, 138.77, 133.80, 139.40, 141.09, 141.66, 154.21, 168.59, 169.25, 171.15. Anal. ($C_{35}H_{28}ClF_3N_4O_6S$) C, H, N.

(4-Chloro-2-trifluoromethyl-phenyl)-carbamic Acid 1-{[3-(4-Methoxy-benzenesulfonyl)-allylcarbamoyl]-methyl}-(3*R*)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-ylmethyl Ester (3d). Compound **12b** (50 mg, 0.085 mmol) was reacted with 4-methoxyphenyl vinyl sulfone (**13d**) (168 mg, 0.85 mmol) according to the same procedure described for **2a**. The title compound was obtained after purification by preparative RP-HPLC (Waters Micromass system, gradient A) as a solid (30.5 mg, 50.7%). RP-UPLC-MS: gradient F, retention time 2.20 min. MS (ESI$^+$) m/z 754.9 [M + H]$^+$ (100%). ^{1}H NMR: 3.84 (s, 3H), 4.00–4.04 (m, 3H), 4.28 (d, J = 15.3 Hz, 1H), 4.59 (d, J = 15.3, 1H), 4.88–5.02 (m, 2H), 6.43 (d, J = 15.0 Hz, 1H), 6.59 (bt, 1H), 6.80 (m, 1H), 6.97 (d, J = 8.8 Hz, 2H), 7.15 (bs, 1H), 7.28 (d, J = 7.5 Hz, 1H), 7.34–7.60 (m, 9H), 7.66 (d, J = 8.4 Hz, 1H), 7.79 (d, J = 8.8 Hz, 2H), 7.99 (d, J = 8.8 Hz, 1H). ^{13}C NMR: 39.32, 52.54, 55.55, 61.73, 65.14, 114.48, 122.51, 124.79, 125.02, 125.23, 126.08, 128.33, 128.98, 129.37, 129.66, 129.85, 130.48, 130.96, 131.28, 131.75, 132.34, 132.73, 137.77, 140.09, 141.96, 153.21, 163.60, 168.19, 169.05, 170.15. Anal. ($C_{36}H_{30}ClF_3N_4O_7S$) C, H, N.

Enzyme Assays. The preliminary screening with FP-2 was performed with 50 μM inhibitor concentrations by using an equivalent amount of DMSO as a negative control. Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC, 32 μM) was determined continuously over a period of 10 min. Since all compounds abolished enzyme activity at this concentration, second-order rate constants of inhibition were determined with seven different inhibitor concentrations, ranging from those that minimally inhibited to those that fully inhibited the enzyme: 0–10 μM for inhibitors **3a** and **2c**; 0–4 μM for inhibitors **2a**, **2b**, **2d**, **3d**, and **E-64**; 0–8 μM for inhibitors **3b** and **3c**. Assays were performed in a 100 mM sodium acetate buffer, pH 5.5 containing 10 mM DTT with Cbz-Phe-Arg-AMC (32 μM) as the substrate.[25] To determine first-order inactivation rate constants (k_{obs}) for the irreversible inhibition, progress curves (fluorescence (F) versus time) were analyzed by nonlinear regression analysis using the equation $F = A(1 - \exp(-k_{obs}t)) + B$.[27] Product formation was monitored continuously for 20 min at room temperature. Fitting of the k_{obs} values against the inhibitor concentrations to the hyperbolic equation $k_{obs} = k_{inac}[I]/(K_{iapp} + [I])$ gave the individual values of K_{iapp} and k_{inac}.[27] The K_{iapp} values were corrected to zero substrate concentration by the term $(1 + [S]/K_m)$ in the equation $K_{inac} = K_{iapp}/(1 + [S]/K_m)$. The second-order rate constants $k_{2nd} = k_{inac}/K_{inac}$ were directly calculated from the individual constants. K_{inac} and k_{inac} values were calculated by nonlinear regression analyses using the program GraFit.[30] The K_m value used to correct the K_{iapp} values was determined to be 21.5 μM.[5a,31] Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed twice in 96-well plates in a total volume of 300 μL. A Varian Cary Eclipse spectrofluorometer (Varian, Darmstadt, Germany) with a microplate reader (excitation at 365 nm, emission at 460 nm) was used. Assays with cathepsins B and L were performed as described previously.[32] Cbz-Phe-Arg-AMC was used as substrate (80 μM for cathepsin B, 5 μM for cathepsin L). The K_m values used to correct K_{iapp} values were 150 μM (cathepsin B) and 6.5 μM (cathepsin L).

Dialysis Assays with Compound 2c. FP-2 (13 μg mL^{-1}) was incubated with **2c** (100 μM) for 5 min. The reaction mixture was then subjected to dialysis against reaction buffer (5000-fold excess) for 3 h. The residual enzyme activities were determined by adding substrate (32 μM). The enzyme was subjected to the same procedure in the absence of inhibitor in order to confirm its stability to the dialysis conditions.

Drug Screening on *P. falciparum* Cultures. The compounds were screened on the human malaria pathogen *P. falciparum* at concentrations between 1 nM and 100 μM. Synchronized ring stages of the *P. falciparum* strain 3D7 were plated in 96-well plates at a parasitemia of 1%, in the presence of the compounds (dissolved in DMSO). Incubation of parasites with DMSO alone at a concentration of 0.5% v/v was used as a negative control. The parasites were cultivated in vitro as described[33] for 72 h. The viability of the parasites was screened subsequently using the Malstat assay, which measures the activity of the *Plasmodium*-specific enzyme lactate dehydrogenase as described.[29]

Acknowledgment. We thank IRBM, MRL Rome for supporting this work and for the generous provision of chemicals. We thank Drs. M. Rowley and V. Summa for valuable scientific discussions and M. C. Palumbi for analytical support. T.S. gratefully acknowledges financial support by the DFG (Deutsche Forschungsgemeinschaft). We would like to thank Prof. Dr. P. Rosenthal, University of California, San Francisco, for supplying us with recombinant FP-2 from *P. falciparum*.

Supporting Information Available: Experimental procedures for the synthesis of compound **10** and analytical data of final compounds **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) WHO. World Malaria Report. http://rbm.who.int./wmr2005/html/exsummary_en.htm (2005).

(2) (a) Mital, A. Recent advances in antimalarial compounds and their patents. *Curr. Med. Chem.* **2007**, *14*, 759–773. (b) Jana, S.; Paliwal, J. Novel molecular targets for antimalarial chemotherapy. *Int. J. Antimicrob. Agents* **2007**, *30*, 4–10.

(3) Wang, S. X.; Pandey, K. C.; Somoza, J. R.; Sijwali, P. S.; Kortemme, T.; Brinen, L. S.; Fletterick, R. J.; Rosenthal, P. J.; McKerrow, J. H. Structural basis for unique mechanisms of folding and hemoglobin binding by a malarial protease. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11503–11508.

(4) Hanspal, M.; Dua, M.; Takakuwa, Y.; Chishti, A. H.; Mizuno, A. *Plasmodium falciparum* cysteine protease Falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. *Blood* **2002**, *100*, 1048–1054.

(5) (a) Shenai, B. R.; Sijwali, P. S.; Singh, A.; Rosenthal, P. J. Characterization of native and recombinant Falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. *J. Biol. Chem.* **2000**, *275*, 29000–29010. (b) Dahl, E. L.; Rosenthal, P. J. Biosynthesis, localization, and processing of falcipain cysteine proteases of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **2005**, *139*, 205–212.

(6) Pandey, K. C.; Sijwali, P. S.; Singh, A.; Na, B. K.; Rosenthal, P. J. Independent intramolecular mediators of folding, activity, and inhibition for the *Plasmodium falciparum* cysteine protease Falcipain-2. *J. Biol. Chem.* **2004**, *279*, 3484–3491.

(7) (a) Leung-Toung, R.; Li, W.; Tam, T. F.; Karimian, K. Thiol-dependent enzymes and their inhibitors: a review. *Curr. Med. Chem.* **2002**, *9*, 979–1002. (b) Ramjee, M. K.; Flinn, N. S.; Pemberton, T. P.; Quibell, M.; Wang, Y.; Watts, J. P. Substrate mapping and inhibitor profiling of falcipain-2, falcipain-3, and berghepain-2: implications for peptidase anti-malarial drug discovery. *Biochem. J.* **2006**, *399*, 47–57.

(8) Ahn, J.-M.; Boyle, N. A.; Macdonald, M. T.; Janda, K. D. Peptidomimetics and peptide backbone modifications. *Mini-Rev. Med. Chem.* **2002**, *2*, 463–473.

(9) Aubé, J. Synthetic routes to lactam peptidomimetics. In *Advances in amino acid mimetics and peptidomimetics.* Abell, A., Ed.; JAI Press: Greenwich, 1997; Vol. 1, pp. 193–232.

(10) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. Emerging approaches in the molecular design of receptor-selective peptide ligands: conformational, topographical and dynamic considerations. *Biochem. J.* **1990**, *268*, 249–262.

(11) Hanessian, S.; McNaughton-Smith, G.; Lombart, H.-G.; Lubell, W. D. Design and synthesis of conformationally constrained acids as versatile scaffolds and peptide mimetics. *Tetrahedron* **1997**, *38*, 12789–12854.

(12) (a) Eguchi, M.; Kahn, M. Design, synthesis, and application of peptide secondary structure mimetics. *Mini-Rev. Med. Chem.* **2002**, *2*, 447–462. (b) Souers, A. J.; Ellman, J. A. β-Turn mimetic library synthesis: scaffolds and applications. *Tetrahedron* **2001**, *57*, 7431–7448. (c) Burgess, K. Solid-phase syntheses of β-turn analogues to mimic or disrupt protein-protein interactions. *Acc. Chem. Res.* **2001**, *34*, 826–835. (d) MacDonald, M.; Aubé, J. Approaches to cyclic peptide β-turn mimics. *Curr. Org. Chem.* **2001**, *5*, 417–438.

(13) (a) Dziadulewicz, E. K.; Brown, M. C.; Dunstan, A. R.; Lee, W.; Said, N. B.; Garratt, P. J. The design of non-peptide human bradykinin B_2 receptor antagonists employing the benzodiazepine peptidomimetic scaffold. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 463–468. (b) Lauffer, D. J.; Mullican, M. D. A practical synthesis of (S) 3-*tert*-butoxycarbonylamino-2-oxo-2,3,4,5-tetrahydro-1,5-benzodiazepine-1-acetic acid methyl ester as a conformationally restricted dipeptido-mimetic for caspase-1 (ICE) inhibitors. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1225–1227.

(14) Micale, N.; Kozikowski, A. P.; Ettari, R.; Grasso, S.; Zappalà, M.; Jeong, J.-J.; Kumar, A.; Hanspal, M.; Chishti, A. H. Novel peptidomimetic cysteine protease inhibitors as potential antimalarial agents. *J. Med. Chem.* **2006**, *49*, 3064–3067.

(15) Shenai, B. R.; Lee, B. J.; Alvarez-Hernandez, A.; Chong, P. Y.; Emal, C. D.; Neitz, R. J.; Roush, W. R.; Rosenthal, P. J. Structure−activity relationships for inhibition of cysteine protease activity and development of *Plasmodium falciparum* by peptidyl vinyl sulfones. *Antimicrob. Agents Chemother.* **2003**, *47*, 154–160.

(16) Palmer, J. T.; Rasnick, D.; Klaus, J. K.; Bromme, D. Vinyl sulfones as mechanism-based cysteine protease inhibitors. *J. Med. Chem.* **1995**, *38*, 3193–3196.

(17) Powers, J. C.; Asgian, J. L.; Ekiei, O. D.; James, K. E. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem. Rev.* **2002**, *102*, 4639–4750.

(18) Vicik, R.; Busemann, M.; Baumann, K.; Schirmeister, T. Inhibitors of cysteine proteases. *Curr. Top. Med. Chem.* **2006**, *6*, 331–353.

(19) Grela, K.; Bieniek, M. Highly selective cross-metathesis with phenyl vinyl sulphone using the 'second generation′ Grubbs' catalyst. *Tetrahedron Lett.* **2001**, *42*, 6425–6428.

(20) (a) Grubbs, R. H.; Chang, S. Recent advances in olefin metathesis and its application in organic synthesis. *Tetrahedron* **1998**, *54*, 4413–4450. (b) Grubbs, R. H. Olefin metathesis. *Tetrahedron* **2004**, *60*, 7117–7140. (c) Trnka, T. M.; Grubbs, R. H. The development of $L_2X_2Ru{=}CHR$ olefin metathesis catalysts: an organometallic success story. *Acc. Chem. Res.* **2001**, *34*, 18–29.

(21) Connon, S. J.; Blechert, S. Recent developments in olefin cross-metathesis. *Angew. Chem., Int. Ed.* **2003**, *42*, 1900–1923.

(22) Sijwali, P. S.; Brinen, L. S.; Rosenthal, P. J. Systematic optimization of expression and refolding of the *Plasmodium falciparum* cysteine protease falcipain-2. *Protein Expression Purif.* **2001**, *22*, 128–138.

(23) Albeck, A.; Paraky, R. Improved stereocontrolled synthesis of *threo* peptididyl epoxides. *J. Org. Chem.* **1994**, *59*, 653–657.

(24) (a) Kingsbury, J. S.; Harrity, J. P. A.; Bonitatebus, P. J.; Hoveyda, A. H. A recyclable Ru-based metathesis catalyst. *J. Am. Chem. Soc.* **1999**, *121*, 791–799. (b) Garber, S. B.; Kingsbury, J. S.; Gray, B. L.; Hoveyda, A. H. Efficient and recyclable monomeric and dendritic Ru-based metathesis catalysts. *J. Am. Chem. Soc.* **2000**, *122*, 8168–8179. (c) Hoveyda, A. H.; Gillingham, D. G.; Van Veldhuizen, J. J.; Kataoka, O.; Garber, S. B.; Kingsbury, J. S.; Harrity, J. P. A. Ru complexes bearing bidentate carbenes: from innocent curiosity to uniquely effective catalysts for olefin metathesis. *Org. Biomol. Chem.* **2004**, *2*, 1–16. (d) Michrowska, A.; Bujok, R.; Harutyunyan, S.; Sashuk, V.; Dolgonos, G.; Grela, K. Nitro-substituted Hoveyda-Grubbs Ruthenium carbenes: enhancement of catalyst activity through electronic activation. *J. Am. Chem. Soc.* **2004**, *126*, 9318–9325.

(25) Bargiggia, F. C.; Murray, W. V. Cross-metathesis assisted by microwave irradiation. *J. Org. Chem.* **2005**, *70*, 9636–9639.

(26) Pandey, K. C.; Wang, S. X.; Sijwali, P. S.; Lau, A. L.; McKerrow, J. H.; Rosenthal, P. J. The *Plasmodium falciparum* cysteine protease falcipain-2 captures its substrate, hemoglobin, via a unique motif. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9138–9143.

(27) Tian, W. X.; Tsou, C. L. Determination of the rate constant of enzyme modification by measuring the substrate reaction in the presence of the modifier. *Biochemistry* **1982**, *21*, 1028–1032.

(28) Schulz, F.; Gelhaus, C.; Degel, B.; Vicik, R.; Heppner, S.; Breuning, A.; Leippe, M.; Gut, J.; Rosenthal, P. J.; Schirmeister, T. Screening of protease inhibitors as antiplasmodial agents. Part I: aziridines and epoxides. *ChemMedChem* **2007**, *2*, 1214–1224.

(29) (a) Goodyer, I. D.; Taraschi, T. F. *Plasmodium falciparum:* a simple, rapid method for detecting parasite clones in microtiter plates. *Exp. Parasitol.* **1997**, *86*, 158–160. (b) Makler, M. T.; Hinrichs, D. J. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am. J. Trop. Med. Hyg.* **1993**, *48*, 205–210. (c) Makler, M. T.; Ries, J. M.; Williams, J. A.;

Bancroft, J E.; Piper, R. C.; Gibbins, B. L.; Hinrichs, D. J. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am. J. Trop. Med. Hyg.* **1993**, *48*, 739–741.

(30) *GraFit*, Version 5.0.1.3; Erithacus Software Ltd.: London, 2006.

(31) Gelhaus, C.; Vicik, R.; Hilgenfeld, R.; Schmidt, C. L.; Leippe, M.; Schirmeister, T. Synthesis and antiplasmodial activity of a cysteine-protease inhibiting biotinylated aziridine-2,3-dicarboxylate. *Biol. Chem.* **2004**, *385*, 435–438.

(32) Vicik, R.; Busemann, M.; Gelhaus, C.; Stiefl, N.; Scheiber, J.; Schmitz, W.; Schulz, F.; Mladenovic, M.; Engels, B.; Leippe, M.; Baumann, K.; Schirmeister, T. Aziridine based inhibitors of cathepsin L—synthesis, inhibition activitiy, and docking studies. *ChemMedChem* **2006**, *1*, 1126–1141.

(33) Ifediba, T.; Vanderberg, J. P. Complete in vitro maturation of *P. falciparum* gametocytes. *Nature* **1981**, *294*, 364–366.

JM701141U