DOI: 10.1002/cmdc.200800050

Nonpeptidic Vinyl and Allyl Phosphonates as Falcipain-2 Inhibitors

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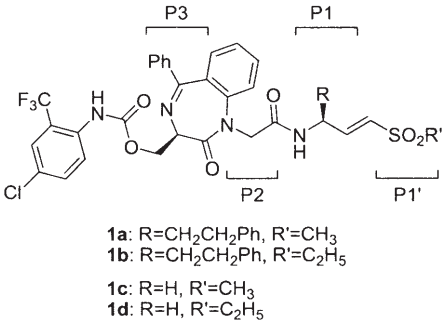
Malaria remains one of the most deadly parasitic diseases, affecting 500 million people all over the world and causing more than one million deaths each year.[1] The limitations of antimalarial chemotherapy underscore the urgent need to discover new drugs that are able to interact with new targets.[2] Research efforts are currently focused on the design of inhibitors of malarial proteases, among which falcipain-2 (FP-2) plays a key role. FP-2 is a papain-family cysteine protease of the most virulent species of the malaria-causing parasite *Plasmodium falciparum*,[3] and is required by mature schizonts for the cleavage of erythrocytic cytoskeletal proteins and by intraerythrocytic trophozoites for hemoglobin degradation, which provides free amino acids for parasite protein synthesis.[4] Thus, selective and irreversible inhibition of FP-2 would be advantageous for the control and elimination of the parasite.[5]

Peptides and peptidomimetics containing an activated double bond, such as vinyl sulfones and vinyl esters, have been shown to be highly potent irreversible cysteine protease inhibitors.[5,6] Whereas the former are active on papain-like enzymes, the latter are known in particular as inhibitors of viral proteases. Both inhibitor types interact with the target enzyme by forming a covalent bond with the thiol group of the active site cysteine. Peptidyl vinyl sulfones are stable and unreactive toward nucleophiles, and require the "catalytic machinery" of cysteine proteases for their activation.[5] Peptidyl vinyl sulfones containing a homoPhe residue at the P1 site have been proven to be highly specific FP-2 inhibitors, with the aromatic side chain being a key structural requirement for greater selectivity toward the target enzyme.[7]

In this context we recently reported a new class of peptidomimetic FP-2 inhibitors based on a rigid benzodiazepine scaffold[8] as a conformationally constrained form of the D-Ser-Gly fragment and on a terminal electrophilic vinyl sulfone moiety on the P1 site that reacts as classical Michael acceptor (such as compounds **1a**–**d**, Figure 1).[9] All the synthesized compounds showed a high level of inhibitory potency and are quite selective toward FP-2, as they weakly inhibit human cysteine proteases cathepsin B and L. In particular, compound **1b** displayed potent enzymatic inhibition ($k_2 = 307\,000\ M^{-1}\,min^{-1}$) coupled

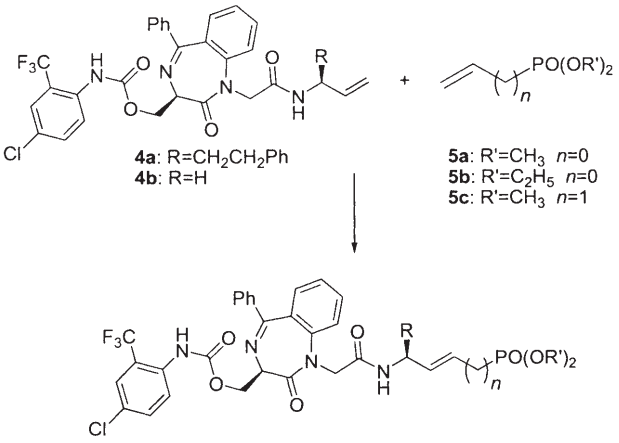


1a: R=CH_2CH_2Ph, R'=CH_3
1b: R=CH_2CH_2Ph, R'=C_2H_5
1c: R=H, R'=CH_3
1d: R=H, R'=C_2H_5

Figure 1. Structure of peptidomimetic FP-2 inhibitors **1a**–**d**.

with a good activity against cultured *P. falciparum* (IC_{50} = 9.1 μM). Among other irreversible cysteine protease inhibitors containing a vinyl moiety conjugated to electron withdrawing groups (EWGs), vinyl phosphonates showed good inhibitory activity against cultured *P. falciparum*.[10]

On this basis, we designed and synthesized nonpeptidic unsaturated phosphonate structures **2** and **3** (Scheme 1) to evaluate their ability to inhibit FP-2 and to make a head-to-head



4a: R=CH_2CH_2Ph
4b: R=H

5a: R'=CH_3 *n*=0
5b: R'=C_2H_5 *n*=0
5c: R'=CH_3 *n*=1

2a: R=CH_2CH_2Ph, R'=CH_3 *n*=0
2b: R=CH_2CH_2Ph, R'=C_2H_5 *n*=0
2c: R=CH_2CH_2Ph, R'=CH_3 *n*=1

3a: R=H, R'=CH_3 n=0
3b: R=H, R'=C_2H_5 *n*=0
3c: R=H, R'=CH_3 *n*=1

Scheme 1. Synthesis of compounds **2** and **3**; reagents and conditions: Hoveyda Catalyst, CH_2Cl_2, 100 °C, MW, 1 h.

comparison with the previously prepared vinyl sulfones **1**. In particular, methyl- and ethylvinyl phosphonate derivatives were synthesized bearing both a homoPhe and a Gly residue in P1 (**2a**–**b** and **3a**–**b**, respectively). Allyl phosphonates **2c** and **3c** were also prepared to compare them with their corresponding vinyl analogues. All compounds were tested on re-

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combinant FP-2[11] and cathepsins B and L to evaluate their inhibitory profile.

The phosphonate inhibitors **2a**–**c** and **3a**–**c** were prepared in excellent yields as *E* stereoisomers via olefin cross-metathesis (CM) employing the Hoveyda–Grubbs 2nd-generation catalyst,[12] by coupling the required vinyl **5a**–**b** or allyl **5c** phosphonates and the tripeptides **4a** or **4b** (Scheme 1), synthesized following a route described previously.[9] Olefin CM represents an alternative to standard olefination methods, with the advantage that a wide range of functional groups is tolerated, the necessary substrate protection is minimal, and the products can be easily prepared with high levels of chemo-, regio-, and stereoselectivity. Furthermore, the concurrent employment of microwave irradiation has revealed several benefits in terms of reaction acceleration together with yield improvement and minimization of by-product formation.[13]

Compounds **2a**–**c** and **3a**–**c** were tested for the inhibition of hydrolysis of the recombinant FP-2 fluorogenic substrate, Cbz-Phe-Arg-AMC (Table 1).[11] An initial screen was performed to find compounds with inhibition >50% at a concentration of 30 μM. For compounds **2a**–**c**, which passed the initial screen, continuous assays (progress curve method)[15] were 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_2 ($M^{-1}min^{-1}$), as $k_2 = k_{inac}/K_{inac}$ (Table 1). In the case of time-independent inhibition (compound **2c**), K_{inac} was determined using a Dixon plot.[16]

Table 1. Inhibition of FP-2 by **1a**–**d**, **2a**–**2c**, and **3a**–**3c**.

Compd	k_2 [$M^{-1}min^{-1}$][a]	k_{inac} [min^{-1}][a]	K_{inac} [μM][a]
1a[b]	432000 ± 63000	0.16 (±0.03)	0.39 (±0.08)
1b[b]	307000 ± 12000	0.10 (±0.05)	0.32 (±0.08)
1c[b]	161000 ± 28000	0.059 (±0.006)	0.37 (±0.15)
1d[b]	248000 ± 22000	0.027 (±0.002)	0.11 (±0.04)
2a	26000 ± 8000	0.08 (±0.01)	2.9 (±1.8)
2b	15000 ± 500	NA[c]	NA[c]
2c	TI[d]	TI[d]	2.63 ± 0.24
3a	no inhibition at 100 μM		
3b	80% inhibition at 100 μM, no inhibition at 30 μM		
3c	30% inhibition at 100 μM, no inhibition at 30 μM		
E-64	1.7×10^6[e]	0.46	0.29

[a] All results include standard deviations from two independent measurements, each performed in duplicate. [b] Data from reference [9]. [c] Plot of k_{obs} versus [I] is restricted to the linear range, thus the individual constants k_{inac} and K_{inac} could not be determined. [d] Time-independent inhibition. [e] $k_{ass} = k_{inac}/K_{iapp} = 11.31$ $(\pm 2.70) \times 10^3\ M^{-1}s^{-1}$; published value[14] with Cbz-Leu-Arg-AMC (25 μM) as substrate: $k_{ass} = 12.28$ $(\pm 1.40) \times 10^3\ M^{-1}s^{-1}$.

Comparison of the results with data obtained for vinyl sulfones **1a**–**d** shows that the potency of the irreversible inhibitors **1**–**3** is strictly dependent on the nature of the Michael acceptor moiety. In this context, the vinyl sulfone moiety in derivatives **1** proves to react better with the active site cysteine; its replacement with the vinyl phosphonate warhead (compounds **2**–**3**) strongly decreases both the inhibitory potency, expressed as the second-order rate of inhibition, and the affinity for the enzyme, evidenced by the dissociation constant (Table 1).

HomoPhe vinyl phosphonates derivatives **2a**–**b** showed higher activity than the corresponding Gly analogues **3a**–**b**. These data indicate the importance of the homoPhe side chain for enzyme inhibition.

The allyl phosphonate **2c**, bearing the EWG group not directly tied to the double bond, presumably interacts with the target enzyme with a mechanism different from classical Michael addition and inhibits the enzyme in a reversible manner. However, the enzyme affinity for both reversible and irreversible inhibitors proved to be similar ($K_{inac} = 2.63$ μM for **2c** versus 2.93 μM for **2a**).

The unsaturated phosphonates **2**–**3** were also tested against the papain-family human cysteine proteases cathepsins B and L (Table 2). Compounds **2a**–**c** proved to be weak reversible inhibitors of cathepsins B and L, with **2a** less potent than **2b**–**c**, showing K_i values one to two orders of magnitude higher than those for FP-2 inhibition, whereas compounds **3a**–**c**, which do not significantly inhibit FP-2, were inactive toward cathepsins B and L. For our class of constrained peptidomimetics, our findings clearly indicate that the vinyl sulfone moiety remains the electrophilic function that interacts better with the FP-2 active site cysteine. These compounds are therefore worthy of further investigations, which are currently underway.

Table 2. Inhibition of cathepsins B and L by **2a**–**2c** and **3a**–**3c**.

Compd	K_{inac} [μM][a]	
	cathepsin B	cathepsin L
2a	>200	49.3 ± 0.3
2b	15.8 ± 2.8	28.6 ± 0.7
2c	19.7 ± 2.3	25.1 ± 3.4
3a	>100	>300
3b	>100	>150
3c	>200	>150

[a] All results include standard deviations from two independent measurements, each performed in duplicate.

Experimental Section

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Reactions under microwave irradiation were performed on a Biotage Emrys Optimizer apparatus. Elemental analyses were carried out on a C. Erba Model 1106 Elemental Analyzer for C, H, and N, and the results were within ±0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC. Preparative HPLC was performed on: 1) an automated (mass-triggered) RP-HPLC Waters Micromass system incorporating a 2525 pump module, a Micromass ZMD detector, and a 2767 collection module operating under Fraction Lynx software. The mobile phase comprised a linear gradient of a binary mixture of MeCN (containing 0.1% TFA) and H_2O (containing 0.1% TFA). The flow rate was 20 mL min^{-1}, the stationary phase was a Symmetry C_{18} column (7 μm, 19×300 mm); gradient A: 50% MeCN for 2 min, 50→90% MeCN over 14 min; gradient B:

40% MeCN for 2 min, 40→90% MeCN over 14 min. 2) a Waters 996 system equipped with a photodiode array detector (PDA). The solvent system was MeCN-0.1% TFA/H_2O-0.1% TFA with a flow rate of 15 mL min^{-1} using an XTerra C_{18} column (5 μm, 19× 100 mm); gradient C: 15% MeCN for 3 min, 15→90% MeCN over 15 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 UPLC BEH C_{18} column (1.7 μm, 2.1× 50 mm); flow rate: 0.5 mL min^{-1}. The solvent system was MeCN-0.1% HCOOH/H_2O-0.1% HCOOH, gradient D: 10→100% MeCN over 0.50 min, then 3 min at 100% MeCN. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM series spectrometer 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).

(3*S*)-(3-{2-[3-(4-Chloro-2-trifluoromethylphenylcarbamoyloxymethyl)-(3*R*)-2-oxo-5-phenyl-2,3-dihydrobenzo[*e*][1,4]diazepin-1-yl]acetylamino}-5-phenylpent-1-enyl)phosphonic acid dimethyl ester (2a). Vinylphosphonic acid dimethyl ester (**5a**) (98 mg, 0.72 mmol) was added to a solution of **4a** (50 mg, 0.072 mmol) in dry CH_2Cl_2 (5 mL), followed by Hoveyda–Grubbs 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 1 h. The solvent was then removed under reduced pressure, and the residue was purified by preparative RP-HPLC (Waters Micromass system, gradient A) to give the title compound as a solid (42 mg, 73%). RP-UPLC–MS: gradient D, $t_R = 2.24$ min; MS (ESI$^+$) *m/z* 797.0 $[M+H]^+$ (100%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.62$–1.95 (m, 2H), 2.50–2.68 (m, 2H), 3.68 (s, 3H), 3.71 (s, 3H), 4.06 (t, $J = 6.5$ Hz, 1H), 4.34 (d, $J = 15.3$ Hz, 1H), 4.52 (d, $J = 15.3$ Hz, 1H), 4.57 (m, 1H) 4.90 (dd, $J = 11.0$, 6.3 Hz, 1H), 5.03 (dd, $J = 11.0$, 6.3 Hz, 1H), 5.74 (bt, $J = 18.4$ Hz, 1H), 6.43 (d, $J = 8.2$ Hz, 1H), 6.70 (m, 1H), 7.01–7.72 (m, 17H), 7.97 ppm (d, $J = 8.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 31.07$, 37.06, 51.90, 53.55, 54.33, 58.35, 65.89, 115.68, 118.59, 121.74, 121.95, 124.32, 124.57, 125.13, 125.98, 127.35, 128.78, 128.80, 128.85, 129.13, 129.45, 129.67, 131.15, 131.65, 131.89, 135.78, 137.17, 138.05, 141.68, 148.86, 155.66, 167.64, 169.85, 170.25 ppm; Anal. calcd for $C_{39}H_{37}ClF_3N_4O_7P$: C 58.76, H 4.67, N 7.03, found: C 58.92, H 4.53, N 6.89.

(3*S*)-(3-{2-[3-(4-Chloro-2-trifluoromethylphenylcarbamoyloxymethyl)-2-oxo-5-phenyl-2,3-dihydrobenzo[*e*][1,4]diazepin-1-yl]-acetylamino}-(3*R*)-5-phenylpent-1-enyl)phosphonic acid diethyl ester (2b). Compound **4a** (50 mg, 0.072 mmol) was reacted with vinylphosphonic acid diethyl ester **5b** (118 mg, 0.72 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 (41 mg, 69%). RP-UPLC–MS: gradient D, $t_R = 2.02$ min; MS (ESI$^+$) *m/z* 825.1 $[M+H]^+$ (100%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.21$–1.30 (m, 6H), 1.70–1.85 (m, 2H), 2.52–2.64 (m, 2H), 3.94–4.08 (m, 5H), 4.23 (d, $J = 14.8$ Hz, 1H), 4.57 (d, $J = 14.8$ Hz, 1H), 4.60 (m, 1H), 4.90 (dd, $J = 11.1$, 6.0 Hz, 1H), 5.08 (dd, $J = 11.1$, 6.0 Hz, 1H), 5.73 (bt, $J = 18.1$ Hz, 1H), 6.48 (d, $J = 8.6$ Hz, 1H), 6.63 (m, 1H), 7.01–7.65 (m, 16H), 7.75 (d, $J = 8.2$ Hz, 1H), 7.90 ppm (d, $J = 8.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.96$, 31.17, 36.96, 52.03, 54.23, 58.68, 62.40, 66.05, 115.98, 119.07, 121.77, 122.07, 124.02, 124.87, 125.43, 126.05, 127.45, 128.67, 128.89, 128.93, 129.23, 129.48, 129.73, 131.25, 131.45, 132.02, 136.07, 137.17, 138.45, 140.98, 149.04, 155.97, 168.02, 169.73, 171.05 ppm; Anal. calcd for $C_{41}H_{41}ClF_3N_4O_7P$: C 59.68, H 5.01, N 6.79, found: C 59.92, H 4.86, N 6.65.

(4*S*)-(4-{2-[3-(4-Chloro-2-trifluoromethylphenylcarbamoyloxymethyl)-(3*R*)-2-oxo-5-phenyl-2,3-dihydrobenzo[*e*][1,4]diazepin-1-yl]acetylamino}-6-phenylhex-2-enyl)phosphonic acid dimethyl ester (2c). Compound **4a** (50 mg, 0.072 mmol) was reacted with allylphosphonic acid dimethyl ester **5c** (108 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 (42 mg, 72%). RP-UPLC–MS: gradient D, $t_R = 1.96$ min; MS (ESI$^+$) *m/z* 811.1 $[M+H]^+$ (100%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.65$–1.80 (m, 2H), 2.48–2.62 (m, 4H), 3.67 (d, $^3J_{PH} = 2.2$ Hz, 3H), 3.71 (d, $^3J_{PH} = 2.2$ Hz, 3H), 4.02 (t, $J = 6.4$ Hz, 1H), 4.27 (d, $J = 15.0$ Hz, 1H), 4.42 (m, 1H), 4.54 (d, $J = 15.0$ Hz, 1H), 4.92 (dd, $J = 11.1$, 6.3 Hz, 1H), 5.06 (dd, $J = 11.1$, 6.3 Hz, 1H), 5.51–5.59 (m, 2H), 6.36 (d, $J = 7.7$ Hz, 1H), 7.06–7.85 (m, 17H), 8.03 ppm (d, $J = 8.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 31.70$, 31.93, 36.16, 50.82, 52.62, 54.10, 61.77, 65.35, 119.83, 119.97, 122.66, 125.07, 125.88, 126.09, 128.25, 128.31, 128.33, 128.35, 129.07, 129.09, 129.11, 129.66, 130.34, 130.85, 132.23, 132.81, 135.19, 137.81, 141.07, 142.07, 142.12, 153.10, 167.36, 169.95, 170.10 ppm; Anal. calcd for $C_{40}H_{39}ClF_3N_4O_7P$: C 59.23, H 4.85, N 6.91, found: C 59.01, H 4.99, N 7.06.

(3-{2-[3-(4-Chloro-2-trifluoromethylphenylcarbamoyloxymethyl)-(3*R*)-2-oxo-5-phenyl-2,3-dihydrobenzo[*e*][1,4]diazepin-1-yl]acetylamino}propenyl)phosphonic acid dimethyl ester (3a). Compound **4b** (50 mg, 0.085 mmol) was reacted with vinylphosphonic acid dimethyl ester **5a** (116 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 (45 mg, 77%). RP-UPLC–MS: gradient D, $t_R = 1.94$ min; MS (ESI$^+$) *m/z* 693.0 $[M+H]^+$ (100%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.68$ (d, $^3J_{PH} = 3.1$ Hz, 3H), 3.72 (d, $^3J_{PH} = 3.3$ Hz, 3H), 3.95–4.05 (m, 2H), 4.08 (t, $J = 6.4$ Hz, 1H), 4.41 (d, $J = 15.5$ Hz, 1H), 4.61 (d, $J = 15.5$ Hz, 1H), 4.94 (dd, $J = 11.3$, 6.6 Hz, 1H), 5.04 (dd, $J = 11.3$, 6.6 Hz, 1H), 5.67 (bt, $J = 17.7$ Hz, 1H), 6.62–6.81 (m, 2H), 7.11 (bs, 1H), 7.26–7.71 (m, 11H), 8.00 ppm (d, $J = 8.84$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 42.84$, 53.52, 54.34, 58.36, 65.88, 114.64, 116.98, 121.76, 121.93, 123.32, 124.55, 125.26, 127.37, 128.99, 129.02, 129.23, 129.45, 130.26, 131.18, 131.35, 132.47, 139.07, 140.75, 146.16, 153.97, 168.68, 170.06, 171.25 ppm; Anal. calcd for $C_{31}H_{29}ClF_3N_4O_7P$: C 53.73, H 4.22, N 8.08, found: C 53.98, H 4.07, N 8.15.

(3-{2-[3-(4-Chloro-2-trifluoromethylphenylcarbamoyloxymethyl)-(3*R*)-2-oxo-5-phenyl-2,3-dihydrobenzo[*e*][1,4]diazepin-1-yl]acetylamino}propenyl)phosphonic acid diethyl ester (3b). Compound **4b** (50 mg, 0.085 mmol) was reacted with vinylphosphonic acid diethyl ester **5b** (140 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 (56 mg, 91%). RP-UPLC–MS: gradient D, $t_R = 2.07$ min; MS (ESI$^+$) *m/z* 721.0 $[M+H]^+$ (100%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.27$–1.33 (m, 6H), 4.00–4.09 (m, 7H), 4.33 (d, $J = 15.5$ Hz, 1H), 4.64 (d, $J = 15.5$ Hz, 1H), 4.95 (dd, $J = 11.2$, 6.5 Hz, 1H), 5.05 (dd, $J = 11.2$, 6.5 Hz, 1H), 5.68 (bt, $J = 19.1$ Hz, 1H), 6.52–6.79 (m, 2H), 7.13 (bs, 1H), 7.30–7.72 (m, 11H), 8.01 ppm (d, $J = 8.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.98$, 42.56, 53.98, 58.24, 62.45, 65.67, 114.45, 116.87, 121.56, 121.79, 123.24, 124.35, 125.12, 127.23, 128.78, 129.12, 129.34, 129.55, 130.34, 131.12, 131.35, 132.51, 139.13, 140.67, 145.23, 152.88, 169.01, 170.12, 171.25 ppm; Anal. calcd for $C_{33}H_{33}ClF_3N_4O_7P$: C 54.97, H 4.61, N 7.77, found: C 54.85, H 4.78, N 7.96.

(4-{2-[3-(4-Chloro-2-trifluoromethylphenylcarbamoyloxymethyl)-(3*R*)-2-oxo-5-phenyl-2,3-dihydrobenzo[*e*][1,4]diazepin-1-yl]acetyl-

amino}but-2-enyl)phosphonic acid dimethyl ester (3c). Compound **4b** (50 mg, 0.085 mmol) was reacted with allylphosphonic acid dimethyl ester **5c** (128 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 (38 mg, 64%). RP-UPLC–MS: gradient D, $t_R = 1.98$ min; MS (ESI^+) m/z 707.3 $[M+H]^+$ (100%); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 2.45$–2.73 (m, 2H), 3.71 (s, 3H), 3.74 (s, 3H), 3.80–3.95 (m, 2H), 4.02 (t, $J = 6.4$ Hz, 1H), 4.32 (d, $J = 15.3$ Hz, 1H), 4.58 (d, $J = 15.3$ Hz, 1H), 4.95 (m, 1H), 5.06 (m, 1H), 5.49–5.78 (m, 2H), 6.42 (bs, 1H), 7.09 (bs, 1H), 7.30–7.71 (m, 11H), 8.06 ppm (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 30.45$, 40.98, 53.24, 54.23, 57.98, 65.67, 114.78, 121.56, 121.89, 123.23, 124.45, 125.01, 127.45, 128.79, 129.12, 129.33, 129.56, 130.23, 131.15, 131.28, 131.34, 132.12, 137.23, 138.79, 140.54, 154.12, 168.55, 170.03, 170.98 ppm; Anal. calcd for $C_{32}H_{31}ClF_3N_4O_7P$: C 54.36, H 4.42, N 7.92, found: C 54.44, H 4.28, N 7.74.

Enzyme assays. The preliminary screening was performed with inhibitor concentrations of 100, 30, and 2 μM using an equivalent amount of DMSO as negative control. Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC, 40 μM) was determined continuously over a period of 10 min. Compounds showing at least 50% inhibition at 30 μM were subjected to detailed assays. These were performed in NaOAc buffer (100 mM pH 5.5) containing DTT (10 mM), with Cbz-Phe-Arg-AMC (40 μM) as substrate.[14] The K_M value used to correct K_{iapp} values was determined at 21.5 μM.[17] 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: 365 nm, emission: 460 nm) was used. To determine first-order inactivation rate constants (k_{obs}) for the time-dependent inhibition, progress curves (fluorescence (F) versus time) for 12 different inhibitor concentrations between 0.05 and 50 μM were analyzed by nonlinear regression analysis using the equation $F = A(1-\exp(-k_{obs}t)) + B$.[15] Product formation was monitored continuously for 15 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}.[15] 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 $k_2 = k_{inac}/K_{inac}$ were directly calculated from the individual constants. K_{inac} and k_{inac} values were calculated by nonlinear regression analyses using the program GraFit.[18] For cases in which the k_{obs} versus [I] plots were restricted to the linear range, k_2 was calculated by using the equation $k_2 \approx k_{obs}[I]^{-1}(1+[S]K_M^{-1})$. In the case of time-independent inhibition, K_{inac} was obtained by a Dixon plot[16] using equation $[E]_0/[E]_a = 1 + [I]/K_{iapp}$ and correction to zero substrate concentration from $K_{inac} = K_{iapp}/(1+[S]K_M^{-1})$ with $[E]_0$ as enzyme activity in the absence, and $[E]_a$ as residual enzyme activities in the presence of the inhibitor. Assays with cathepsins B and L were performed as described previously.[19] 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).

Acknowledgements

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

Keywords: cysteine proteases · falcipain-2 inhibitors · Michael acceptors · vinyl phosphonates

[1] World Malaria Report, *W.H.O.* **2005**: http://rbm.who.int./wmr2005/html/exsummary_en.htm.
[2] a) S. Jana, J. Paliwal, *Int. J. Antimicrob. Agents* **2007**, *30*, 4–10; b) G. E. Garcia Linares, J. B. Rodriguez, *Curr. Med. Chem.* **2007**, *14*, 289–314.
[3] P. J. Rosenthal, P. S. Sijwali, A. Singh, B. R. Shenai, *Curr. Pharm. Des.* **2002**, *8*, 1659–1672.
[4] a) M. Hanspal, M. Dua, Y. Takakuwa, A. H. Chishti, A. Mizuno, *Blood* **2002**, *100*, 1048–1054; b) T. Hogg, K. Nagarajan, S. Herzberg, L. Chen, X. Shen, H. Jiang, M. Wecke, C. Blohmke, R. Hilgenfeld, C. L. Schmidt, *J. Biol. Chem.* **2006**, *281*, 25425–25437.
[5] J. C. Powers, J. L. Asgian, O. D. Ekici, K. E. James, *Chem. Rev.* **2002**, *102*, 4639–4650.
[6] R. Vicik, M. Busemann, K. Baumann, T. Schirmeister, *Curr. Top. Med. Chem.* **2006**, *6*, 331–353.
[7] B. R. Shenai, B. J. Lee, A. Alvarez-Hernandez, P. Y. Chong, C. D. Emal, R. J. Neitz, W. R. Roush, P. J. Rosenthal, *Antimicrob. Agents Chemother.* **2003**, *47*, 154–160.
[8] N. Micale, A. P. Kozikowski, R. Ettari, S. Grasso, M. Zappalà, J.-J. Jeong, A. Kumar, M. Hanspal, A. H. Chishti, *J. Med. Chem.* **2006**, *49*, 3064–3067.
[9] R. Ettari, E. Nizi, M. E. Di Francesco, M.-A. Dude, G. Pradel, R. Vičík, T. Schirmeister, N. Micale, S. Grasso, M. Zappalà, *J. Med. Chem.* **2008**, *51*, 988–996.
[10] J. T. Palmer, D. Rasnick, J. L. Klaus, Pct. Int. Appl. WO9523222, **1995** [*Chem. Abstr.* **1995**, *123*, 309464].
[11] P. S. Sijwali, L. S. Brinen, P. J. Rosenthal, *Protein Expression Purif.* **2001**, *22*, 128–138.
[12] A. K. Chatterjee, T.-L. Choi, R. H. Grubbs, *Synlett* **2001**, 1034–1037.
[13] F. C. Bargiggia, W. V. Murray, *J. Org. Chem.* **2005**, *70*, 9636–9639.
[14] K. C. Pandey, S. X. Wang, P. S. Sijwali, A. L. Lau, J. H. McKerrow, P. J. Rosenthal, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 9138–9143.
[15] W. X. Tian, C. L. Tsou, *Biochemistry* **1982**, *21*, 1028–1032.
[16] P. J. F. Henderson, *Biochem. J.* **1972**, *127*, 321–333.
[17] C. Gelhaus, R. Vicik, R. Hilgenfeld, C. L. Schmidt, M. Leippe, T. Schirmeister, *Biol. Chem.* **2004**, *385*, 435–438.
[18] GraFit Version 3.0, Erithacus Software Ltd., London, **1992**.
[19] R. Vicik, M. Busemann, C. Gelhaus, N. Stiefl, J. Scheiber, W. Schmitz, F. Schulz, M. Mladenovic, B. Engels, M. Leippe, K. Baumann, T. Schirmeister, *ChemMedChem* **2006**, *1*, 1126–1141.

Received: February 21, 2008
Revised: April 1, 2008
Published online on April 21, 2008

