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Screening of Protease Inhibitors as Antiplasmodial Agents. Part I: Aziridines and Epoxides

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A broad protease-based and cell-based screening of protease inhibitors yielded the aziridine-2-carboxylic acid derivative ***2a*** *and the* N*-acylated aziridine-2,3-dicarboxylic acid derivatives* ***32a*** *and* ***34b*** *as the most potent inhibitors of falcipain-2 and falcipain-3 (IC_{50} falcipain-2: 0.079–5.4 μM, falcipain-3: 0.25–39.8 μM). As the compounds also display* in vitro *activity against the* P. falciparum *parasite in the submicromolar and low micromolar range, these compound classes are leads for new antiplasmodial falcipain inhibitors.*

Introduction

Malaria, caused by the protozoan parasite *Plasmodium*, is one of the most serious infectious diseases worldwide. Among the species causing malaria in humans, *Plasmodium falciparum* is the most virulent, causing more than one million deaths each year, mostly in children under five years old. The increasing resistance[1,2] of malaria parasites to antimalarial drugs, the lack of highly effective vaccines, and inadequate control of mosquito vectors results in the problem growing, especially in the developing world.[3,4] Thus, new approaches to drug development are needed.

Proteases of malaria parasites play pivotal roles in the processes of host erythrocyte rupture, erythrocyte invasion, and hemoglobin degradation.[5] Among the *Plasmodium* proteases, cysteine proteases are well studied and characterized.[6,7] Treatment with cysteine protease inhibitors blocks hemoglobin hydrolysis and development of the parasite.[8–10] Therefore, the inhibition of cysteine proteases presents a promising strategy for combating the infection. The best characterized plasmodial cysteine proteases are the falcipains, which are papain-like enzymes belonging to clan CA, family C1: falcipain-1, falcipain-2, falcipain-2′, and falcipain-3.[11,12] Whereas falcipain-1 is suggested to play a principal role in nonerythrocytic stages (for example, falcipain-1 knock out decreases oocyst production in the sexual cycle),[13] falcipain-2 and falcipain-3 are likely the major hemoglobinases in the food vacuole of erythrocytic parasites.[14]

To identify new potential agents against *P. falciparum* we screened 88 compounds against falcipain-2, falcipain-3, and cultured *P. falciparum*. The scrutinized compound classes comprise electrophilic compounds, namely aziridine-2-carboxylic acid derivatives (35 compounds in total),[15] aziridine-2,3-dicarboxylic acid derivatives (42 compounds in total),[16–18] and corresponding epoxides (11 compounds in total),[19] all known to inhibit proteases by covalent reaction. In addition, one corresponding compound lacking the three-membered ring (TMR) was also tested.

Results and Discussion

Syntheses

The syntheses of the compounds (Scheme 1–6) are summarized briefly as follows:

N-Alkylated aziridine-2-carboxylic acid derivatives with free 3-position **1–4** (Table 1) and **13** (Table 3) were obtained either by reaction of 2-bromo acrylic acid derivatives or by reaction of corresponding dibromo propanoic acid derivatives with amino acids or amines (Scheme 1).[15]

The *N*-benzyl-3-methyl- or −3-phenyl aziridine-2-carboxylic acid derivatives **5**–**11** (Table 1) were synthesized by Cromwell synthesis starting from crotonic (compound **5**) or cinnamic

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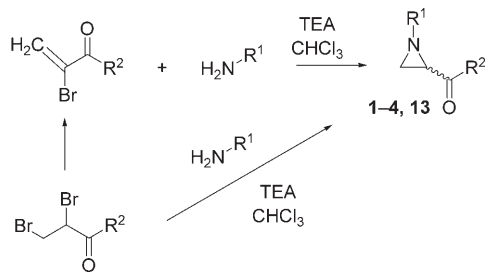


Scheme 1. Synthesis of *N*-alkylated aziridine-2-carboxylic acid derivatives with free 3-position (**1**–**4**), and (**13**); TEA, triethylamine.

acid esters (**6**–**11**) (Scheme 2) (leading to the ethyl aziridine-2-carboxylic acid derivatives), ester hydrolysis, and subsequent peptide coupling.[19]

The *cis*-configured *N*-benzyl aziridine-2,3-dicarboxylic acid derivative **12** (Table 1, Scheme 3) was synthesized starting from the *anti*-bromo alcohol (synthesized from L-tartrate), S_N2 reaction with sodium azide yielding the *syn*-azido alcohol, ring closure reaction (Staudinger reaction under Mitsunobu conditions) to the diethyl aziridine-2,3-dicarboxylate, *N*-benzylation, ester hydrolysis to the half ester, and subsequent peptide coupling with PheOBn.[19]

The aziridine-2,3-dicarboxylic acid derivatives **29**–**40** (Table 2) were prepared by *N*-acylation of diethyl or dibenzyl *trans*-aziridine-2,3-dicarboxylates with amino acids or dipeptides (Scheme 4).[17]

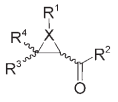
The epoxide **23** (Table 1) was synthesized (Scheme 5) starting from maleic acid, epoxidation, ester hydrolysis, and peptide coupling.[19]

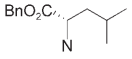
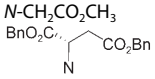
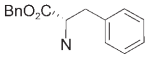
The epoxides **24** (Table 1, Table S2) were synthesized by glycide ester synthesis (Scheme 6), ester hydrolysis, peptide coupling, and final chromatographic separation of the diastereomers **24a** and **24b**.[19]

Biological evaluation

Falcipain-2 and falcipain-3 were recombinantly expressed in *E. coli* and refolded to active enzyme as previously described.[20] IC_{50} values for inhibition of falcipain-2 and falcipain-3 were determined as described previously using the fluorogenic substrates Cbz-Phe-Arg-AMC and Cbz-Leu-Arg-AMC, respectively (AMC, 7-amino-4 methylcoumarin).[21–23] For the most active compounds k_i, K_i, and k_{2nd} values were determined according to the continuous assay method.[24,25] As a positive control the well-known cysteine protease inhibitor E-64,[21,25,26] and as negative control the solvent DMSO was used. Dose-dependent effects of compounds on parasite development (*P. falciparum* strain W2) were quantified by flow cytometry according to a previously published method.[12] First, a screening with inhibitor concentrations of 100 μM, 10 μM, and 1 μM was performed and the percentage activity of infected red blood cells (RBCs) relative to the negative control was determined. Compounds showing concentration dependent inhibition in these assays were selected for determination of IC_{50} values (Tables 1–3). Chloroquine[27] and again E-64[28] were used as positive controls, and the solvent DMSO was used as a negative control. For selected compounds additional assays with the *P. falciparum* strain FCBR were preformed using a fluorometric assay with Hoechst-33258,[29] and/or the microculture tetrazolium test measuring parasite lactate dehydrogenase activity.[30]

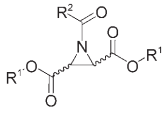
Table 1. Inhibition of falcipains (FP) and antiplasmodial activity of aziridine- and oxirane-2-carboxylates **1**–**12**, **23**–**24**.



Compd.	X-R^1	R^2	R^3/R^4	Configuration TMR	FP 2 IC_{50} [μM]	FP 3 IC_{50} [μM]	*P. falc.* IC_{50} [μM][b]
1a		OCH_3	H/H	*R*+*S* (9)[a]	33.1	57.9	21.9
2a	*N*-$CH_2CO_2CH_3$	(*S*)-PheOBn	H/H	*R*+*S* (0)[a]	2.2	3.8	9.7
3		(*S*)-PheOMe	H/H	*R*+*S* (0)[a]	29.2	64.2	16.7
4a		OCH_3	H/H	*R*+*S* (20)[a]	59.9	n.i.	28.0
5[c]	*N*-Benzyl	(*S*)-PheOBn	Me/H	*R,R*+*S,S* (0)[a]	92.8	n.i.	57.1
6	*N*-Benzyl	(*S*)-PheOBn	Phenyl/H	*R,R*+*S,S* (4.7)[a]	84.7	n.i.	10.8
7	*N*-Benzyl	(*S*)-ValOBn	Phenyl/H	*R,R*+*S,S* (16)[a]	51.1	n.i.	3.2
8[d]	*N*-Benzyl	(*S*)-Phe-(*S*)-Ala-(*S*)-LeuOBn	Phenyl/H	*R,R*+*S,S* (53)[a]	14.6	41.1	13.1
9	*N*-Benzyl	(*S*)-Phe-(*S*)-Leu-(*S*)-Ala-(*S*)-ProOMe	Phenyl/H	*R,R*+*S,S* (68)[a]	38.7	84.4	22.7
10	*N*-Benzyl	(*S*)-Pro-(*S*)-LeuOBn	Phenyl/H	*R,R*+*S,S* (45)[a]	36.3	n.i.	40.7
11	*N*-Benzyl	(*S*)-*N*-CH(Benzyl)-CH_2OH	Phenyl/H	*R,R or S,S*	115.8	n.i.	48.4
12	*N*-Benzyl	(*S*)-PheOBn	EtO_2C/H	*S,R*+*R,S* (26)[a]	38.4	n.i.	29.9
23	O	(*S*)-Phe-(*S*)-LeuOBn	EtO_2C/H	*R,S*+*S,R* (18)[a]	39.2	47	27.3
24a	O	(*S*)-PheOBn	Me/Phenyl	*R,S*+*S,R* (0)[a]	52.0	111	n.i.

[a] diastereomeric excess (%); [b] determined by flow cytometry, strain W2; [c] the single diastereomer with (*S,S*)-configured aziridine ring is equipotent against falcipains and *P. falciparum*; [d] the enriched diastereomer (de = 82%) with reversed configuration at the aziridine ring exhibits the following IC_{50} values: falcipain-2: 21.3 μM/falcipain-3: 101.3 μM/*P. falciparum*: 16.9 μM. n.i.: no inhibition at 100 μM.

Table 2. Inhibition of falcipains (FP) and antiplasmodial activity of aziridine-2,3-dicarboxylates **29**–**40**.



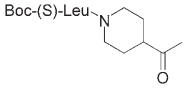
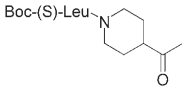
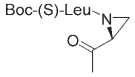
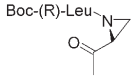
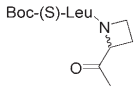
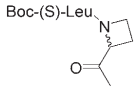
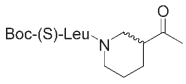
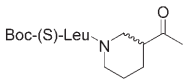
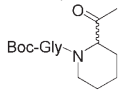
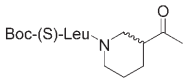
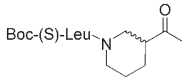
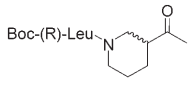
Compd.	R^1	R^2-C(=O)	Configuration aziridine	FP 2 IC_{50} [μM]	FP 3 IC_{50} [μM]	*P. falc.* IC_{50} [μM]
29	Et	Cbz-(*S*)-Leu-	*R,R*	2.0	n.i.	41.8[c,d]
30a	Et	Boc-(*S*)-Leu-(*S*)-Pro-	*S,S*	216	515	27.5[d]
30b	Et	Boc-(*S*)-Leu-(*S*)-Pro-	*R,R*	n.i.	n.i.	n.i.
31a	Et		*S,S*	95.7	n.d.	51.5[d]
31b	Et		*R,R*	n.i.	17	n.i.
32a	Bn		*S,S*	0.079/1.4[a]	0.25	0.43[d]/7.6[c]/5.1[b]
32b	Bn		*S,S*	152	n.i.	n.i.
33a	Et		*R,R*	133	n.i.	10.4[c]
33b	Bn		*S,S*	164	88.8	8.6[d]
34a	Bn	Boc-(*R*)-Leu-(*S*)-Pro-	*S,S*	135	75.6	16.7[d]
34b	Bn	Boc-(*S*)-Leu-(*S*)-Pro-	*R,R*	5.4[e]	39.8	0.78[d]/1.8[c]/4.2[b]
34c	Bn	Boc-(*S*)-Leu-(*R*)-Pro-	*S,S*	209	149	17.5[c]/16.2[b]
34d	Bn	Boc-(*R*)-Leu-(*R*)-Pro-	*S,S*	n.i.	287	7.1[c]
34e	Bn	Boc-(*S*)-Leu-(*S*)-Pro-	*S,S*	n.i.	83.1	8.9[c]
35a	Bn	Boc-(*S*)-Phe-(*R*)-Ala-	*S,S*	22.4[f]	73.8	4.2[d]
35b	Bn	Boc-(*R*)-Phe-(*S*)-Ala	*S,S*	n.i.	102	n.i.
35c	Bn	Boc-(*R*)-Phe-(*R*)-Ala	*S,S*	45	52	n.i.
35d	Bn	Boc-(*S*)-Phe-(*S*)-Ala-	*S,S*	n.i.	171	9.3[c]
36a	Et		*S,S*	61.9	n.i.	n.i.
36b	Et		*R,R*	133	556	n.i.
37a	Bn	Boc-Gly-(*S*)-Pro-	*S,S*	n.i.	104	n.i.
37b	Bn	Boc-Gly-(*R*)-Pro-	*S,S*	447	87	n.i.
38	Bn		*S,S*	n.i.	n.i.	n.i.
39a	Bn		*S,S*	171	78	n.i.
39b	Bn		*S,S*	228	74	n.i.
40	Bn		*S,S*	228	50	n.i.
E-64				0.015[g,h]	0.075 [h]	5.3[c,i]

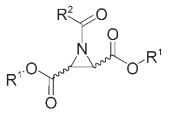
Table 1 (aziridine-2-carboxylic acid derivatives and respective epoxides) and Table 2 (*N*-acylated aziridine-2,3-dicarboxylic acid derivatives) show the activities of the compounds against falcipains and/or *P. falciparum*. Table 3 contains compounds of the aziridine-2-carboxylic acid derivative series containing a Lys residue. These compounds do not or only moderately inhibit falcipains, but are highly active (IC_{50} 0.5–9.9 μM) against the parasite in vitro. A list of all other compounds tested (**1b**, **2b**, **4b**, **14**–**22**, **24b**, **25**–**28**, **41**–**53**) can be found in the Supporting Information (Tables S1–S5). These compounds inhibit neither falcipains nor *P. falciparum*.

In the following, the results will be analyzed with respect to: 1) the structure–activity relationship (SAR) of the inhibition of falcipains, and 2) the correlation between falcipain inhibition and antiplasmodial activity.

1. SAR of falcipain-2 and falcipain-3 inhibition

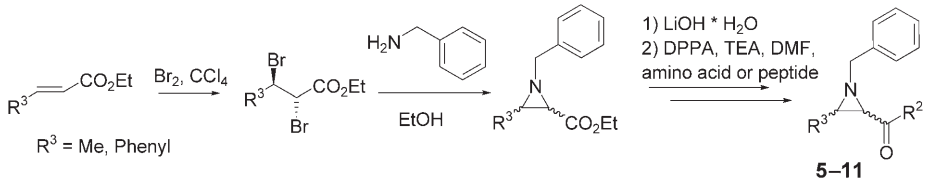
There are several compounds within the series of aziridine-2-carboxylic acid derivatives with free 3-position ($R^3 = R^4 = H$), namely compounds **1a**, **2a**, **3**, **4a** (Table 1), and **13** (Table 3), which exhibit inhibition of falcipains. Among these, inhibitor **2a** is most active with IC_{50} values of 2.2 μM (falcipain-2) and 3.8 μM (falcipain-3), whereas the others only moderately inhibit both enzymes ($IC_{50} >$ 21 μM). Notably, compounds **1a**, **2a**, **3**, and **4a** contain quite hydrophobic amino acid benzyl esters (LeuOBn, PheOBn, or Asp-$(OBn)_2$). In contrast, corresponding methyl esters (Table S1: compounds **1b** (LeuOMe), **2b** (PheOMe), **4b** (PheOMe), **19** (PheOMe), **21** (PheOMe)), and Asp derivatives **18a**–**c** lacking the PheOMe moiety at C2 do

Table 2. (Continued)



Compd.	R^1	R^2-C(=O)	Configuration aziridine	FP 2 IC_{50} [μM]	FP 3 IC_{50} [μM]	*P. falc.* IC_{50} [μM]
Chloroquin				n.d.	n.d.	0.24[b]

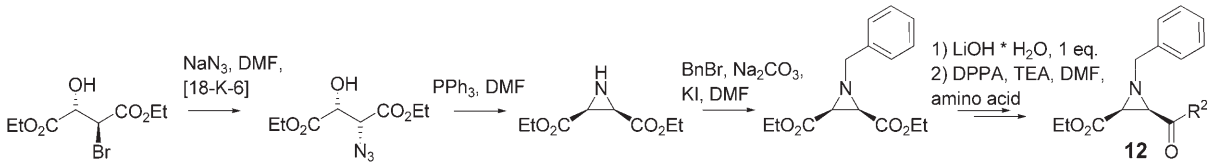
The diastereomeric excess of compounds **33a**, **33b**, **36a**, **36b**, **38**, **39a**, **39b**, **40** is 0%; n.d., not determined; n.i., no inhibition at 100 μM; [a] determined after 5 min incubation of enzyme and inhibitor prior to substrate addition; continuous assay results: $k_i = 0.02\ min^{-1}$, $K_i = 0.2$ μM, $k_{2nd} = 95{,}238\ M^{-1}min^{-1}$; [b] determined by flow cytometry, strain W2; [c] determined with Hoechst-33258, strain FCBR; [d] determined by measuring lactate dehydrogenase activity, strain FCBR; [e] continuous assay results: $k_i = 0.02\ min^{-1}$, $K_i = 0.4$ μM, $k_{2nd} = 51{,}282\ M^{-1}min^{-1}$; [f] continuous assay results: $k_i = 0.03\ min^{-1}$, $K_i = 6.5$ μM, $k_{2nd} = 4{,}615\ M^{-1}min^{-1}$; [g] $k_{2nd} = 1.7(\pm0.4)\times10^6\ M^{-1}min^{-1}$, $k_i = 0.46(\pm0.066)\ min^{-1}$, $K_i = 0.29\ (\pm0.089)$ μM, literature values: $k_{ass} = 12.28\ (\pm1.40)\times10^3\ M^{-1}s^{-1}$ (ref. [25]), $k_{2nd} = 1.16\times10^3\ M^{-1}s^{-1}$ (ref. [26]); [h] literature values: falcipain-2: 0.012 μM, falcipain-3: 0.032 μM (ref. [23]);[i] literature value with strain W2: 3.2 μM (ref. [28]).



Scheme 2. Synthesis of *N*-benzyl-3-methyl (**5**) or -3-phenyl aziridines (**6**–**11**); TEA, triethylamine; DPPA, diphenylphosphoryl azide.

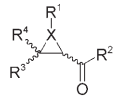
not inhibit the enzymes. Furthermore, aziridine-2-carboxylic acid derivatives derived from less hydrophobic amino acid esters GlyOR, AlaOR, ValOR, MetOMe (Table S1: **14**, **15**, **16**, **17**), and derivative **22** (Table S1) containing the space-filling trityl fragment attached to the aziridine nitrogen also do not inhibit falcipains.

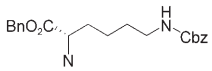
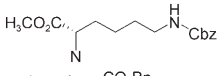
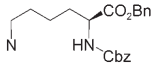
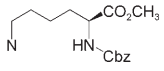
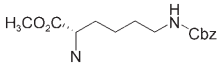
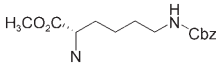
Within the *N*-benzylated aziridine series (Table 1) containing either a phenyl ring (**6**–**11**), a methyl group (**5**), or an ethyl ester group (**12**) at C3 of the TMR only one compound exhibits considerable inhibition of falcipain-2, namely inhibitor **8** ($IC_{50} = 14.6$ μM) containing the tripeptide Phe-Ala-LeuOBn at C2. Compounds with shorter sequences (**6**, **12**: PheOBn, **11**: phenylalaninol, **7**: ValOBn, **10**: Pro-LeuOBn), and the compound with the tetrapeptide



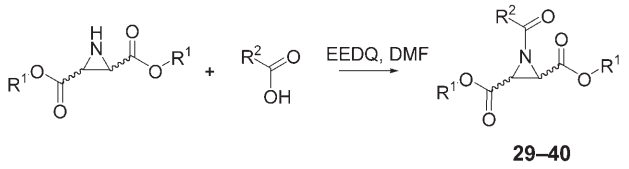
Scheme 3. Synthesis of the *N*-benzylated aziridine-2,3-dicarboxylic acid derivative (**12**); TEA, triethylamine; DPPA, diphenylphosphoryl azide.

Table 3. Inhibition of falcipains (FP) and antiplasmodial activity of aziridine-2-carboxylates **13**, containing a Lys residue.

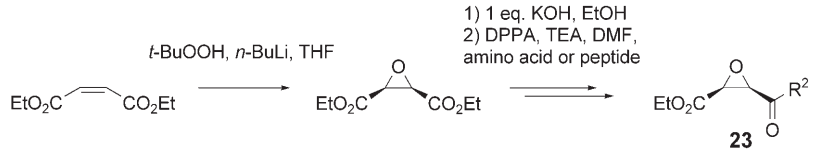


Comp.	X-R^1	R^2	R^3/R^4	Configuration TMR	FP 2 IC_{50} [μM]	FP 3 IC_{50} [μM]	*P. falc.* IC_{50} [μM][b]
13a		OCH_3	H/H	*R*+*S* (0)[a]	21.9	67.9	4.5
13b		OCH_3	H/H	*R*+*S* (0)[a]	51.0	n.i.	0.5
13c		OCH_3	H/H	*R*+*S* (0)[a]	59.8	n.i.	2.2
13d		OCH_3	H/H	*R*+*S* (13)[a]	n.i.	n.i.	9.8
13e		(*S*)-PheOMe	H/H	*R*+*S* (0)[a]	46.8	n.i.	1.7
13f		(*S*)-Asp$(OBn)_2$	H/H	*R*+*S* (0)[a]	21.0	n.i.	5.5

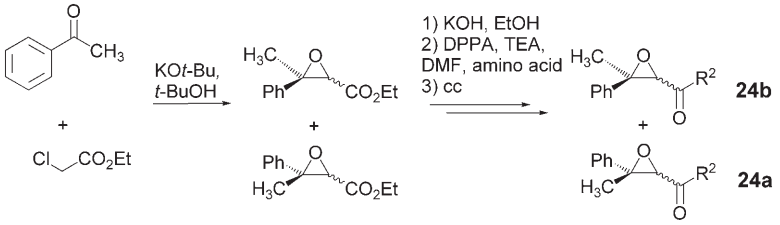
[a] diastereomeric excess (%); [b] determined by flow cytometry, strain W2. n.i.: no inhibition at 100 μM.



Scheme 4. Synthesis of *N*-acylated aziridine-2,3-dicarboxylic acid derivatives (**29**–**40**); EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.



Scheme 5. Synthesis of epoxide (**23**); TEA, triethylamine; DPPA, diphenylphosphoryl azide.



Scheme 6. Synthesis of epoxides (**24**); TEA, triethylamine; DPPA, diphenylphosphoryl azide; cc, column chromatography.

Phe-Leu-Ala-ProOBn (**9**) at C2 are only moderately active. Inhibition of falcipain-3 is weaker than inhibition of falcipain-2, a phenomenon that is also observed with most other inhibitors tested here.

Within the tested epoxide series only the derivative containing an additional phenyl ring at C3 (**24a**), and the dipeptide derivative **23** show moderate activity against both enzymes. Moreover, the diastereomer of **24a**, namely **24b**, which contains the phenyl ring in *cis*-position to the amino acid at C2 is inactive (Table S2).

Within the series of *N*-acylated aziridine-2,3-dicarboxylic acid derivatives (Table 2, Table S3) only one compound containing a sole amino acid instead of a dipeptide moiety attached to the aziridine nitrogen (**29**) exhibits falcipain-2 inhibition whereas it is nearly inactive against falcipain-3. All other derivatives within this subseries (Table S3: **41**–**45**) inhibit neither falcipain-2 nor falcipain-3. Notably, compound **29** is the only derivative containing Cbz-Leu, whereas all others contain a Boc-protected cyclic amino acid attached to the aziridine nitrogen. Comparing compound **30a** with the corresponding dibenzyl ester **34e** it became apparent that the weak falcipain-2 inhibition is lost whereas the inhibition of falcipain-3 is enhanced sixfold. Similarly, the dibenzyl ester **33b** is a better falcipain-3 inhibitor than the diethyl ester **33a**, **39a** is more active than **36a**, and **34b** is superior than its diethyl ester counterpart **30b** in terms of falcipain-3 inhibition. **34b** displays the highest inhibition potency (falcipain-2: $IC_{50} = 5.4\ \mu M$; falcipain-3: $IC_{50} = 39.8\ \mu M$) within the subseries of Boc-Leu-Pro containing derivatives (**30**, **34**). Compounds containing Gly (**37**, **40**) instead of Leu (**39**, **34**) show similar potency against both enzymes. Within the Phe-Ala series (**35a–d**) **35a** with a (*S*)-Phe-(*R*)-AlaOBn moiety displays the highest activity (falcipain-2, $IC_{50} = 22.4\ \mu M$). The most active compound against both falcipains is inhibitor **32a** which contains an additional aziridine moiety. With an IC_{50} value of 0.079 μM against falcipain-2 and an IC_{50} value of 0.25 μM against falcipain-3, the compound displays very good inhibition potency. The diastereomer with (*R*)-Leu (**32b**) instead of (*S*)-Leu only weakly inhibits falcipain-2, and is inactive against falcipain-3. A detailed inspection of the inhibition constants of **32a** ($K_i = 0.2\ \mu M$, $k_i = 0.02\ min^{-1}$, $k_{2nd} = 95{,}238\ M^{-1}min^{-1}$) showed in agreement with previous results[16,17] that the compound displays a low alkylation rate constant. Thus, the remarkable inhibition is mainly due to high affinity to the enzyme, and not to a fast alkylation rate. The different IC_{50} values against falcipain-2 measured on the one hand without preincubation of enzyme with inhibitor prior to substrate addition (1.4 μM), and on the other hand with 30 min preincubation time (0.079 μM) reflect the time-dependent inhibition normally displayed by irreversible inhibitors. Previously performed assays with cathepsin L and B,[17] and rhodesain[16] showed this compound (**32a**) to be also the most potent cathepsin L and rhodesain inhibitor within the aziridine-2,3-dicarboxylic acid derivative series ($K_i = 13\ nM$ for cathepsin L, $K_i = 0.3\ \mu M$ for rhodesain). With selectivity indices of 723 (cathepsin L), 31 (rhodesain), and 47 (falcipain-2) versus cathepsin B ($K_i = 9.4\ \mu M$) **32a** is selective for the cathepsin-L like cysteine proteases of clan CAC1. Thus, the proposed binding mode on cathepsin L,[17] which predicts binding into S and S′ subsites may also apply to the other related enzymes.

The data revealed also a good correlation concerning inhibition of related proteases within the Leu-Pro series (**34**): **34b** is the most active inhibitor against cathepsin L,[17] rhodesain[16] and falcipains.

Aziridine-2,3-dicarboxylic acid derivatives or -2,2-dicarboxylic acid derivatives with free aziridine nitrogen (Table S3: **46**–**51**) do neither inhibit falcipains nor *P. falciparum*. The same holds true for the oxirane-2,2-dicarboxylic acid derivative **51**, and the succinic acid derivative **53** which lacks a TMR.

2. Inhibition of P. falciparum

A variety of compounds exhibit IC_{50} values against *P. falciparum* below or equal to 10 μM: **2a**, **6**, **7**, **32a**, **33a**, **33b**, **34b**, **34d**, **34e**, **35a**, **35d**, and the Lys containing inhibitors **13a–f**.

Among these, the following inhibitors potently inhibit both enzymes, falcipain-2 and falcipain-3: **2a**, **32a**, **34b**. In contrast, compound **29**, which displays high falcipain-2 inhibition (IC_{50} = 2 μM) while being inactive against falcipain-3, is only moderately active against the parasite.

Inhibitors **6**, **7**, **33a**, **33b**, **34d**, **34e**, and **35d** only moderately inhibit one or both of the target enzymes or are completely inactive. For these compounds, and also for the Lys containing compounds (**13**) (with the most active antiplasmodial derivative **9b**, IC_{50} = 0.5 μM) other targets than falcipain-2 and falcipain-3 or additional targets have to be considered. As the *cis*-configured aziridines and epoxides (**5**–**12**, **23**), and among these especially compounds **6** and **7** are known to inhibit aspartic acid proteases[19] the plasmodial plasmepsins (I, II, IV)[31] can be considered as additional targets. In the case of compounds **6** and **7**, plasmepsin-II inhibition[19] was already excluded. However, *P. falciparum* also expresses several other aspartic proteases[31] which might be targets for these compounds.

For the most active *P. falciparum* inhibitors among the compounds lacking high falcipain inhibition (**7**, **13b)** unselective cytotoxicity as reason for the high antiplasmodial activity was also excluded (IC_{50} against human macrophages: > 100 μM). Thus, these compounds are highly interesting in terms of target search.

It is worth mentioning that the *N*-acylated aziridine-2,3-dicarboxylic acid derivatives displaying high antiplasmodial activity are also found to display trypanocidal activity, with inhibitor **34b** being the most active one against *Trypanosoma b. brucei* (IC_{50} = 10 μM).[16] This inhibitor which is less toxic than **32a** (for example, human macrophages: IC_{50} > 125 μM for **34b** versus 50 μM for **32a**)[16] could be a new promising lead for cysteine protease inhibitors displaying both antiplasmodial and antitrypanosomal activity. On the other hand, the inhibitors **34a** and **34c** with IC_{50} values around 16 μM against *P. falciparum* display very good antileishmanial activity while being nontoxic against various human cells.[18]

Comparing inhibition of two different *P. falciparum* strains which was investigated with the two most active compounds **32a** and **34b**, no significant differences could be found. A comparison of the three different assay methods (FACS analysis after labeling with YOYO-1 dye, fluorometric assay with Hoechst-33258, microculture tetrazolium test) which was also undertaken with these two compounds (**32a**, **34b**) shows that the assay with MTT gave lower IC_{50} values whereas the other two assays gave results similar to each other.

Conclusions

A broad protease- and cell-based screening of 88 compounds containing a TMR as reactive moiety revealed the aziridine-2-carboxylic acid derivative **2a**, and the *N*-acylated aziridine-2,3-dicarboxylic acid derivatives **32a** and **34b** as most active inhibitors of falcipain-2 (IC_{50} = 2.2/0.079/5.4 μM) and falcipain-3 (IC_{50} = 3.8/0.25/39.8 μM), concomitantly displaying high antiplasmodial activity (IC_{50} = 9.7/5.1/4.2 μM). These compounds are highly promising concerning further development as antiplasmodial falcipain inhibitors for several reasons: compounds **2a** and **32a** because of their potent falcipain-3 inhibition; **34b** because of its high additional potency against rhodesain and *T. b. brucei* without toxicity to human cells.[16] In addition, this compound shows neither cross-reactivity against proteases of other classes (for example, serine proteases), nor reactivity against low-molecular weight thiols (for example, DTT, dithiothreitol).

The data for the best falcipain inhibitors within the series of *N*-acylated aziridine-2,3-dicarboxylic acid derivatives (**32a**, **34b**) correlate well to those previously obtained for other cathepsin-L like cysteine proteases indicating similar binding modes, namely binding to both nonprimed and primed substrate binding pockets. According to the recently resolved X-ray structure of falcipain-2[37] selective falcipain-2 inhibitors may be designed taking advantage of the more restricted S2-pocket of falcipain-2 compared to other cathepsin-L like cysteine proteases, leading to preference of Leu versus Phe. In addition, these studies[37] showed that primed-site interactions comparable to those seen in the falcipain-2/cystatin complex[37] could also be exploited for enhancing selectivity. In this regards **34b** is a good lead for further improvement of affinity and selectivity.

On the other hand, several antiplasmodial compounds (IC_{50} 0.5–10 μM) were identified for which additional and/or other targets than falcipain-2 or falcipain-3 have to be considered. For these compounds a broad range cytotoxicity, for example, against human cells, or inhibition of plasmepsin-II can be excluded already.

Experimental Section

General

ESI mass spectra were recorded on a FT-ICR mass spectrometer APEX II, Bruker. NMR spectra were recorded on an AVANCE 400 MHz spectrometer from Bruker Biospin GmbH, Germany (solvent: $CDCl_3$, ^1H NMR: 400.13 MHz; ^{13}C NMR: 100.61 MHz). IR spectra were recorded on a PharmalyzIR FT-IR spectrometer from BioRad, USA. α values were determined on a 241 polarimeter from PerkinElmer, USA. LC-MS: Agilent 1100 LC/MSD-Trap, HPLC-System 1100 Agilent; Phenomenex Jupiter 4μ Proteo 90 A RP C-18 column (4.6× 150 mm), gradient:

time (min)	solvent B (%)
0	20
10	70
15	40

solvent A: water, 0.1% formic acid; solvent B: acetonitrile, 0.1% formic acid, detection: 254 nm.

Syntheses

The following compounds were prepared as described recently: aziridine-2,3-dicarboxylic acid derivatives (**29**–**40**, **41**–**45**) in ref. [17], aziridine-2-carboxylic acid derivatives (**1a**, **2b**, **13d**, **14a**, **14b**, **15a**, **16a**, **16b**, **17**, **18b**) in ref. [15], aziridine-2-carboxylic acid derivatives (**5**–**12**) in ref. [19], oxiranes (**23**–**28**) in ref. [19], the aziridine- and oxirane-2,2-dicarboxylic acid derivatives (**51**–**52**) in ref. [32], the succinic acid derivative **53** in ref. [19]. Cpd. **22** was synthesized according to ref. [33]. All amino acids used are L-configured.

Methyl 1-((*S*)-1-methoxycarbonyl-3-methyl-butyl)-aziridine-2-carboxylic acid derivative (**1b**) was synthesized as mixture of diaste-

reomers (de = 0%) as described earlier for compound **1a**,[15] namely by reaction of methyl-2-bromo acrylate with LeuOMe. The diastereomers were not separated. Yield: 78%; IR: $\tilde{\nu}$ = 2956, 2872, 1734, 1439, 1197, 1170 753; diastereomer 1: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.82–1.71 (m, 8H, CH_3, ^{5}CH, $^4CH_2$), 1.74–1.84 (m, 2H, $^4CH_2$, $^1CH_2$), 2.07–2.10 (m, 1H, ^{2}CH), 2.22–2.36 (m, 2H, $^1CH_2$, ^{3}CH), 3.65, 3.66 ppm (s, je 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.35, 22.85 (CH_3), 24.80 (^{5}CH), 33.85 ($^1CH_2$), 37.27 (^{2}CH), 41.52 ($^4CH_2$), 51.96, 52.24 (OCH_3), 70.10 (^{3}CH), 169.56, 171.00 ppm (C=O); diastereomer 2: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.82–1.71 (m, 9H, CH_3, $^1CH_2$, ^{5}CH, $^4CH_2$), 1.74–1.84 (m, 1H, $^4CH_2$), 2.22–2.36 (m, 3H, $^1CH_2$, ^{2}CH, ^{3}CH), 3.65, 3.66 ppm (s, je 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.41, 22.91 (CH_3), 24.75 (^{5}CH), 33.47 ($^1CH_2$), 36.42 (^{2}CH), 41.52 ($^4CH_2$), 51.96, 52.24 (OCH_3), 69.59 (^{3}CH), 169.56, 171.22 ppm (C=O). LOOP-ESI-MS: calcd. f. $C_{11}H_{19}NO_4$, 229.28; found: $[M+H]^+$ 230.3. LC-MS: R_t = 1.0 min, purity: > 99%; $[\alpha]_D^{26}$ = +17 ($CHCl_3$, c = 0.1).

Benzyl (*S*)-2-[(1-methoxycarbonylmethyl-aziridine-2-carbonyl)-amino]-3-phenyl propionate (**2a**) was synthesized as a mixture of diastereomers (de = 0%) as described earlier for compound **2b**,[15] namely by reaction of benzyl-2[(2-bromoacryloyl)amino]-3-phenylpropanoate with GlyOMe. The diastereomers were not separated. Yield: 26%; IR: $\tilde{\nu}$ = 2962, 2919, 2849, 1736, 1452, 1349, 1204, 1174, 741, 697; diastereomer 1: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.57–1.61 (m, 1H, $^1CH_2$), 1.73 (d, 1H, $^1CH_2$, $^3J_{1B,2}$ = 3.29 Hz), 2.03 (dd, 1H, ^{2}CH, $^3J_{2,1A/B}$ = 3.29, 6.82 Hz), 2.83–3.29 (m, 4H, $^3CH_2$, $^5CH_2$), 3.66 (s, 3H, OCH_3), 4.77–4.85 (m, 1H, ^{4}CH), 5.00–5.14 (m, 2H, $^6CH_2$), 5.97 (bs, 1H, NH), 7.14–7.29 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 33.21 ($^1CH_2$), 36.91 ($^5CH_2$), 38.38 (^{2}CH), 50.99 (OCH_3), 53.20 (^{4}CH), 58.40 ($^3CH_2$), 66.07 ($^6CH_2$), 125.98, 126.26, 127.43, 128.25, 128.31 (CH, arom.), 133.93 (C_q, arom.), 167.59, 169.60, 170.09 ppm (C=O); diastereomer 2: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.57–1.61 (m, 1H, $^1CH_2$), 1.92 (d, 1H, $^1CH_2$, $^3J_{1B,2}$ = 3.28 Hz), 2.06 (dd, 1H, ^{2}CH, $^3J_{2,1A/B}$ = 3.28, 6.83 Hz), 2.83–3.29 (m, 4H, $^3CH_2$, $^5CH_2$), 3.67 (s, 3H, OCH_3), 4.77–4.85 (m, 1H, ^{4}CH), 5.00–5.14 (m, 2H, $^6CH_2$), 5.97 (bs, 1H, NH), 7.14–7.29 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 33.40 ($^1CH_2$), 36.61 ($^5CH_2$), 38.44 (^{2}CH), 50.94 (OCH_3), 51.49 (^{4}CH), 58.40 ($^3CH_2$), 66.46 ($^6CH_2$), 125.98, 126.26, 127.43, 128.25, 128.31 (CH, arom.), 133.93 (C_q, arom.), 167.59, 169.60, 170.09 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{22}H_{24}N_2O_5$, 396.45; found: $[M+H]^+$ 397.5; LC-MS: R_t = 1.6 min, purity: 93.1%; $[\alpha]_D^{26}$ = −14 ($CHCl_3$, c = 0.1).

Dibenzyl (*S*)-2-[2-((*S*)-1-methoxycarbonyl-2-phenyl-ethylcarbamoyl)-aziridine-1-yl] succinate (**3**) was synthesized as previously described,[15] namely by reaction of benzyl-2[(2-bromoacryloyl)amino]-3-phenylpropanoate with dibenzyl aspartate ($Asp(OBn)_2$). Yield: 11% (mixture of diastereomers which were not separated, de = 0%); IR: $\tilde{\nu}$ = 3063, 3031, 2952, 1733, 1452, 1357, 1209, 1172, 742, 697; diastereomer 1: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.67 (d, 1H, $^1CH_2$, $^3J_{1A,2}$ = 7.33 Hz), 1.85 (d, 1H, $^1CH_2$, $^3J_{1B,2}$ = 3.54 Hz), 2.33 (dd, 1H, ^{2}CH, $^3J_{2,1A/B}$ = 3.54, 7.33 Hz), 2.67–3.11 (m, 5H, ^{3}CH, $^4CH_2$, $^6CH_2$), 3.57 (s, 3H, OCH_3), 4.65–4.70 (m, 1H, ^{5}CH), 4.97–5.08 (m, 4H, $^7CH_2$), 6.97 (bs, 1H, NH), 7.00–7.31 ppm (m, 15H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 36.86 ($^1CH_2$), 37.13 ($^6CH_2$), 37.85 ($^4CH_2$), 39.16 (^{2}CH), 52.32 (OCH_3), 52.40 (^{5}CH), 65.82 (^{3}CH), 66.85 ($^7CH_2$), 127.85, 128.35, 128.43, 128.49, 128.62, 128.72, 129.27, 129.31 (CH, arom.), 135.18, 135.42, 135.78 (C_q, arom.), 169.03, 170.06, 171.52, 171.63 ppm (C=O); diastereomer 2: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.74 (d, 1H, $^1CH_2$, $^3J_{1A,2}$ = 3.28 Hz), 1.88 (d, 1H, $^1CH_2$, $^3J_{1B,2}$ = 7.07 Hz), 2.12 (dd, 1H, ^{2}CH, $^3J_{2,1A/B}$ = 3.28, 7.07 Hz), 2.67–3.11 (m, 5H, ^{3}CH, $^4CH_2$, $^6CH_2$), 3.57 (s, 3H, OCH_3), 4.65–4.70 (m, 1H, ^{5}CH), 4.97–5.08 (m, 4H, $^7CH_2$), 6.97 (bs, 1H, NH), 7.00–7.31 ppm (m, 15H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 33.85 ($^1CH_2$), 37.13 ($^6CH_2$), 37.85 ($^4CH_2$), 38.55 (^{2}CH), 52.15 (OCH_3), 52.40 (^{5}CH), 65.92 (^{3}CH), 67.27 ($^7CH_2$), 127.85, 128.35, 128.43, 128.49, 128.62, 128.72, 129.27, 129.31 (CH, arom.), 135.18, 135.42, 135.78 (C_q, arom.), 169.03, 170.06, 171.52, 171.63 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{31}H_{32}N_2O_7$, 544.61; found: $[M+H]^+$ 545.5; LC-MS: R_t = 4.6 min, purity: 94.9%; $[\alpha]_D^{26}$ = −33 ($CHCl_3$, c = 0.1).

Methyl 1-((*S*)-1-methoxycarbonyl-2-phenyl-ethyl)-aziridine-2-carboxylic acid derivative (**4b**) was synthesized as previously described,[15] namely by reaction of methyl-2-bromo acrylate with PheOMe. The diastereomers (de = 20%) were not separated. Yield: 85%; IR: $\tilde{\nu}$ = 3062, 3028, 2953, 2847, 1736, 1672, 1454, 1350, 1200, 1175, 748; diastereomer 1: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.18 (d, 1H, $^1CH_2$, $^3J_{1A,2}$ = 6.57 Hz), 1.66–1.72 (m, 1H, ^{2}CH), 2.01 (d, 1H, $^1CH_2$, $^3J_{1B,2}$ = 3.28 Hz), 2.45–2.49 (m, 1H, ^{3}CH), 2.78–2.83 (m, 1H, $^4CH_2$), 3.15–3.11 (m, 1H, $^4CH_2$), 3.58, 3.66 (s, 6H, OCH_3), 7.11–7.26 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 33.05 ($^1CH_2$), 38.28 (^{2}CH), 38.97 (CH_2Ph), 52.01, 52.23 (OCH_3), 73.07 (^{3}CH), 125.84, 127.52, 128.27 (arom.), 136.32 (arom., C_q), 169.22, 170.19 ppm (C=O); diastereomer 2: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.66–1.72 (m, 1H, $^1CH_2$), 2.15–2.18 (m, 2H, $^1CH_2$, ^{2}CH), 2.45–2.49 (m, 1H, ^{3}CH), 2.78–2.83 (m, 1H, $^4CH_2$), 3.15–3.11 (m, 1H, $^4CH_2$), 3.65, 3.67 (s, 6H, OCH_3), 7.11–7.26 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 34.49 ($^1CH_2$), 36.38 (^{2}CH), 41.00 ($^4CH_2$), 52.09, 52.25 (OCH_3), 73.30 (^{3}CH), 126.85, 127.58, 129.28 (arom.), 137.15 (arom., C_q), 169.58, 174.27 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{14}H_{17}NO_4$, 263.30; found: $[M+H]^+$ 264.3; LC-MS: R_t = 1.5 min, purity: 98.2%; $[\alpha]_D^{26}$ = −13 ($CHCl_3$, c = 0.1).

Methyl 1-((*S*)-1-benzyloxycarbonyl-5-benzyl oxycarbonylamino-pentyl)-aziridine-2-carboxylic acid derivative (**13a**) was synthesized as described recently for compound **13d**,[15] namely from methyl-2,3-dibromopropanoate and *N*ε-Cbz-LysOBn. The diastereomers (1/1) were not separated. Yield: 40%; IR: $\tilde{\nu}$ = 3063, 3034, 2951, 2862, 1716, 1439, 1343, 1200, 1178, 737, 697; diastereomer 1: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.18–1.41 (m, 4H, $^6CH_2$, $^5CH_2$), 1.59–1.80 (m, 3H, $^1CH_2$, $^4CH_2$), 2.20–2.25 (m, 3H, $^1CH_2$, ^{2}CH, ^{3}CH), 3.06–3.10 (m, 2H, $^7CH_2$), 3.58 (s, 3H, OCH_3), 5.01–5.11 (m, 4H, $^8CH_2$), 7.24–7.28 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 21.70 ($^5CH_2$), 28.34 ($^6CH_2$), 31.02 ($^4CH_2$), 32.97 ($^1CH_2$), 36.34 (^{2}CH), 39.69 ($^7CH_2$), 51.28 (OCH_3), 65.58 ($^8CH_2$), 70.35 (^{3}CH), 127.08, 127.44, 127.50, 127.55, 127.59, 127.64 (arom., CH), 134.55, 134.64 (arom., C_q), 155.36, 169.49, 169.87 ppm (C=O); diastereomer 2: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.18–1.41 (m, 4H, $^6CH_2$, $^5CH_2$), 1.59–1.80 (m, 3H, $^1CH_2$, $^4CH_2$), 2.02–2.04 (m, 1H, ^{2}CH), 2.20–2.25 (m, 2H, $^1CH_2$, ^{3}CH), 3.06–3.10 (m, 2H, $^7CH_2$), 3.58 (s, 3H, OCH_3), 5.01–5.11 (m, 4H, $^8CH_2$), 7.24–7.28 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 21.70 ($^5CH_2$), 28.34 ($^6CH_2$), 30.83 ($^1CH_2$), 31.02 ($^4CH_2$), 35.89 (^{2}CH), 39.69 ($^7CH_2$), 51.28 (OCH_3), 65.58 ($^8CH_2$), 70.35 (^{3}CH), 127.08, 127.44, 127.50, 127.55, 127.59, 127.64 (arom., CH), 134.55, 134.64 (arom., C_q), 155.36, 169.49, 169.87 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{25}H_{30}N_2O_6$, 454.53; found: $[M+H]^+$ 455.5; LC-MS: R_t = 1.8 min, purity: 93%; $[\alpha]_D^{26}$ = +21 ($CHCl_3$, c = 0.1).

Methyl 1-((*S*)-5-benzyloxycarbonylamino-1-methoxycarbonyl-pentyl)-aziridine-2-carboxylic acid derivative (**13b**) was synthesized as previously described,[15] from methyl 2,3-dibromopropanoate and *N*ε-Z-LysOMe. The diastereomers (1/1) were not separated. Yield: 23%; IR: $\tilde{\nu}$ = 2949, 2865, 1699, 1442, 1335, 1242, 1175, 738, 697; diastereomer 1: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.29–1.47 (m, 4H, $^6CH_2$ und $^5CH_2$), 1.60 (m, 1H, $^1CH_2$), 1.75–1.84 (m, 2H, $^4CH_2$), 2.06–2.09 (m, 1H, ^{2}CH), 2.20–2.26 (m, 2H, $^1CH_2$ und ^{3}CH), 3.06–3.13 (m, 2H, $^7CH_2$), 3.65 (s, 3H, OCH_3), 3.66 (s, 3H, OCH_3), 4.80 (bs, NH), 5.01 (s, 2H, $^8CH_2$), 7.21–7.28 ppm (m, 5H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 22.75 ($^5CH_2$), 31.89 ($^6CH_2$), 33.80 ($^4CH_2$), 35.26 ($^1CH_2$), 37.35 (^{2}CH), 40.71 ($^7CH_2$), 52.15, 52.37 (OCH_3), 66.57 ($^8CH_2$), 71.43 (^{3}CH), 128.07, 128.08, 128.49, (arom., CH), 136.65 (arom., C_q), 156.37, 170.47, 171.57 ppm (C=O); diastereomer 2: ^{1}H NMR ($CDCl_3$,

400 MHz): $\delta = 1.29–1.47$ (m, 4H, $^6CH_2$ und $^5CH_2$), 1.75–1.84 (m, 3H, $^4CH_2$ und $^1CH_2$), 2.20–2.26 (m, 3H, $^1CH_2$, ^2CH und ^3CH), 3.06–3.13 (m, 2H, $^7CH_2$), 3.64 (s, 3H, OCH_3), 3.66 (s, 3H, OCH_3), 4.80 (bs, NH), 5.01 (s, 2H, $^8CH_2$), 7.21–7.28 ppm (m, 5H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 22.75$ ($^5CH_2$), 31.89 ($^6CH_2$), 33.80 ($^4CH_2$), 35.47 ($^1CH_2$), 36.86 (^2CH), 40.20 ($^7CH_2$), 52.15, 52.37 (OCH_3), 66.57 ($^8CH_2$), 71.31 (^3CH), 128.07, 128.08, 128.49, (arom., CH), 136.65 (arom., C_q), 156.37, 170.47, 171.57 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{19}H_{26}N_2O_6$, 378.4; found: $[M]^+$ 378.3; LC-MS: $R_t = 1.1$ min, purity: 87.1%; $[\alpha]_D^{26} = +5$ ($CHCl_3$, $c = 0.1$).

Methyl 1-((*S*)-5-benzyloxycarbonyl-5-benzyloxycarbonylamino-pentyl)-aziridine-2-carboxylic acid derivative (**13c**) was synthesized as previously described,[15] from methyl 2,3-dibromopropanoate and *N*α-Z-LysOBn. The diastereomers (1/1) were not separated. Yield: 40%; IR: $\tilde{\nu} = 2951$, 2862, 1716, 1438, 1334, 1204, 1178, 740, 698; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.20–2.24$ (m, 11H, $^6CH_2$, $^5CH_2$, $^4CH_2$, $^3CH_2$, ^2CH und $^1CH_2$), 3.61 (s, 3H, OCH_3), 4.28–4.34 (m, 1H, ^7CH), 5.01–5.12 (m, 4H, $^8CH_2$), 5.38 (bs, 1H, NH), 7.21–7.26 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta =$ 22.85 ($^5CH_2$), 28.95 ($^4CH_2$), 32.38 ($^6CH_2$), 34.59 ($^1CH_2$), 37.21 (^2CH), 52.18 (OCH_3), 53.87 (^7CH), 60.50 ($^3CH_2$), 66.93, 67.08 ($^8CH_2$), 128.09, 128.14, 128.31, 128.46, 128.51, 128.62 (CH, arom.), 135.37, 136.30 (C_q, arom.), 155.91 (HNC=O), 171.30, 172.25 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.20–2.24$ (m, 11H, $^6CH_2$, $^5CH_2$, $^4CH_2$, $^3CH_2$, ^2CH und $^1CH_2$), 3.61 (s, 3H. OCH_3), 4.28–4.34 (m, 1H, ^7CH), 5.01–5.12 (m, 4H, $^8CH_2$), 5.38 (bs, 1H, NH), 7.21–7.26 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 22.94$ ($^5CH_2$), 29.00 ($^4CH_2$), 32.41 ($^6CH_2$), 34.61 ($^1CH_2$), 37.25 (^2CH), 52.18 (OCH_3), 53.87 (^7CH), 60.53 ($^3CH_2$), 66.93, 67.08 ($^8CH_2$), 128.09, 128.14, 128.31, 128.46, 128.51, 128.62 (CH, arom.), 135.37, 136.30 (C_q, arom.), 155.91 (HNC=O), 171.30, 172.25 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{25}H_{30}N_2O_6$, 454.53; found: $[M+Na]^+$ 477.5; LC-MS: $R_t = 2.1$ min, purity: 91.1%; $[\alpha]_D^{26} = -56$ ($CHCl_3$, $c = 0.1$).

Methyl (*S*)-6-benzyloxycarbonylamino-2-[2-(1-methoxycarbonyl-2-phenyl-ethylcarbamoyl)-aziridine-1-yl]-hexanoate (**13e**) was synthesized from methyl 2-[(2-bromoacryloyl)amino]-3-phenylpropanoate and *N*ε-Cbz-LysOMe.[15] The diastereomers (1/1) were not separated. Yield: 21%; IR: $\tilde{\nu} = 2951$, 2863, 1736, 1437, 1355, 1214, 1177, 742, 699; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.18–1.47$ (m, 4H, $^5CH_2$, $^6CH_2$), 1.57 (d, 1H, $^1CH_2$, $^3J_{3A,2} = 7.08$ Hz), 1.65–1.76 (m, 2H, $^4CH_2$), 1.91 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.03$ Hz), 2.13 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.03$, 7.08 Hz), 2.27 (m, 1H, ^3CH), 3.01–3.11 (m, 4H, $^7CH_2$, $^{10}CH_2$), 3.52, 3.58 (s, 3H, OCH_3), 4.68–4.81 (m, 1H, ^9CH), 5.02 (s, 2H, $^8CH_2$), 5.17 (bs, 1H, NH), 6.97–7.28 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 22.64$ ($^5CH_2$), 31.95 ($^6CH_2$), 33.92 ($^4CH_2$), 37.67 ($^1CH_2$), 37.81 ($^{10}CH_2$), 38.60 (^2CH), 40.78 ($^7CH_2$), 51.99, 52.43 (OCH_3), 59.23 (^9CH), 66.63 ($^8CH_2$), 70.01 (^3CH), 127.08, 128.11, 129.36 (CH, arom.), 135.87, 136.76 (C_q, arom.), 156.41, 169.37, 171.22, 172.16 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.18–1.47$ (m, 4H, $^5CH_2$, $^6CH_2$), 1.65–1.76 (m, 3H, $^1CH_2$, $^4CH_2$), 1.78 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.28$ Hz), 1.97–1.99 (m, 1H, ^2CH), 2.27 (m, 1H, ^3CH), 3.01–3.11 (m, 4H, $^7CH_2$, $^{10}CH_2$), 3.57, 3.66 (s, 3H, OCH_3), 4.68–4.81 (m, 1H, ^9CH), 5.00 (s, 2H, $^8CH_2$), 5.17 (bs, 1H, NH), 6.97–7.28 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta =$ 22.51 ($^5CH_2$), 31.86 ($^6CH_2$), 34.85 ($^4CH_2$), 37.93 ($^1CH_2$), 38.66 ($^{10}CH_2$), 38.60 (^2CH), 40.70 ($^7CH_2$), 52.07, 52.20 (OCH_3), 59.23 (^9CH), 66.56 ($^8CH_2$), 70.01 (^3CH), 128.49, 128.53, 129.11 (CH, arom.), 135.91, 136.65 (C_q, arom.), 156.52, 169.51, 171.73, 171.84 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{28}H_{35}N_3O_7$, 525.61; found: $[M+H]^+$ 526.5; LC-MS: $R_t = 1.5$, 1.9 min, purity: 94.9%; $[\alpha]_D^{26} = +11$ ($CHCl_3$, $c = 0.1$).

2-(*S*)-{[1-(5-Benzyloxycarbonylamino-1-methoxycarbonyl-pentyl)-aziridine-2-carbonyl]-amino}-succinic acid dibenzyl ester (**13f**) was synthesized from dibenzyl 2-[(2-bromoacryloyl)amino]succinate and *N*ε-Cbz-LysOMe.[15] The diastereomers (1/1) were not separated. Yield: 17%; IR: $\tilde{\nu} = 3064$, 3033, 2950, 2864, 1730, 1454, 1354, 1211, 1174, 749, 696; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.30–1.47$ (m, 4H, $^5CH_2$, $^6CH_2$), 1.57 (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.83$ Hz), 1.67–1.75 (m, 2H, $^4CH_2$), 1.91 (d, 1H, $^1CH_2$, $^3J_{1B,2} =$ 3.03 Hz), 2.11 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.03$, 6.83 Hz), 2.28–2.32 (m, 1H, ^3CH), 2.80–3.04 (m, 2H, $^{10}CH_2$), 3.05–3.13 (m, 1H, ^7CH), 3.54 (s, 3H, OCH_3), 4.75–4.82 (m, 1H, ^9CH), 4.94–5.00 (m, 6H, $^8CH_2$, $^{11}CH_2$), 7.14–7.30 ppm (m, 15H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 22.75$ (^5CH), 31.89 ($^6CH_2$), 33.70 ($^4CH_2$), 36.38 ($^7CH_2$), 36.48 ($^1CH_2$), 38.54 (^2CH), 40.85 ($^{10}CH_2$), 48.15 (^9CH), 52.09 (OCH_3), 66.52, 66.54, 67.67 ($^8CH_2$, $^{11}CH_2$), 70.09 (^3CH), 128.10, 128.17, 128.27, 128.44, 128.48, 128.52 (CH, arom.), 135.21, 135.42, 136.65 (C_q, arom.), 156.42, 169.61, 170.40, 171.91 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.30–1.47$ (m, 4H, $^5CH_2$, $^6CH_2$), 1.67–1.75 (m, 3H, $^1CH_2$, $^4CH_2$), 1.89 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.03$ Hz), 2.05 (dd, 1H, ^2CH, $^3J_{2,1A/B} =$ 3.03, 7.07 Hz), 2.28–2.32 (m, 1H, ^3CH), 2.80–3.04 (m, 2H, $^{10}CH_2$), 3.05–3.13 (m, 2H, $^7CH_2$), 3.54 (s, 3H, OCH_3), 4.75–4.82 (m, 1H, ^9CH), 4.94–5.00 (m, 6H, $^8CH_2$, $^{11}CH_2$), 7.14–7.30 ppm (m, 15H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 22.75$ (^5CH), 31.89 ($^6CH_2$), 33.70 ($^4CH_2$), 36.38 ($^7CH_2$), 36.48 ($^1CH_2$), 38.54 (^2CH), 40.85 ($^{10}CH_2$), 48.05 (^9CH), 51.96 (OCH_3), 66.52, 66.54, 67.67 ($^8CH_2$, $^{11}CH_2$), 69.88 (^3CH), 128.10, 128.17, 128.27, 128.44, 128.48, 128.52 (CH, arom.), 135.21, 135.42, 136.65 (C_q, arom.), 156.42, 169.61, 170.40, 171.91 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{37}H_{45}N_3O_9$, 675.79; found: $[M+Na]^+$ 699.6; LC-MS: $R_t = 1.3$, 1.9 min, purity: 92.5%; $[\alpha]_D^{26} = +2$ ($CHCl_3$, $c =$ 0.1).

Methyl ((*S*)-1-benzyloxycarbonyl-ethyl)-aziridine-2-carboxylic acid derivative (**15b**) was synthesized as described for compound **15a**,[15] namely by reaction of methyl-2-bromo acrylate with AlaOBn. The diastereomers (de = 11%) were not separated. Yield: 88%; IR: $\tilde{\nu} = 3063$, 3033, 2953, 2891, 1732, 1455, 1357, 1211, 1165, 737, 697; diastereomer 1: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.37$ (d, 3H, $^4CH_3$, $^3J = 6.82$ Hz), 1.58 (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.57$ Hz), 2.08 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.16$, 6.57 Hz), 2.20 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.16$ Hz), 2.26–2.32 (m, 1H, ^3CH), 3.58 (s, 3H, OCH_3), 5.06–5.15 (m, 2H, CH_2Ph), 7.24–7.29 ppm (m, 5H, arom.); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.11$ ($^4CH_3$), 34.86 ($^1CH_2$), 36.57 (^2CH), 52.37 (OCH_3), 66.69 (^3CH), 66.78 (CH_2Ph), 128.29, 128.33, 128.59 (CH, arom.), 135.64 (C_q, arom.), 170.70, 171.48 ppm (C=O); diastereomer 2: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.38$ (d, 3H, $^4CH_3$, $^3J = 6.82$ Hz), 1.82 (d, 1H, $^1CH_2$, $^3J_{1A,2} =$ 6.57 Hz), 2.26–2.32 (m, 3H, ^3CH, ^2CH und $^1CH_2$), 3.66 (s, 3H, OCH_3), 5.06–5.15 (m, 2H, CH_2Ph), 7.24–7.29 ppm (m, 5H, arom.); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.15$ ($^4CH_3$), 33.00 ($^1CH_2$), 38.22 (^2CH), 52.28 (OCH_3), 66.67 (^3CH), 66.86 (CH_2Ph), 128.18, 128.24, 128.54 (CH, arom.), 135.55 (C_q, arom.), 170.45, 171.23 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{14}H_{17}NO_4$, 263.3; found: $[M+H]^+$ 264.5; LC-MS: $R_t = 0.9$, 1.1 min, purity: >99%; $[\alpha]_D^{26} = +4$ ($CHCl_3$, $c = 0.1$).

18a and **18c** were synthesized as previously described,[15] namely by reaction of methyl-2-bromo acrylate with either dimethyl (**18a**) or di-*tert*-butyl aspartate (**18c**).

Dimethyl (*S*)-2-(2-methoxycarbonyl-aziridine-2-yl)-succinate (**18a**): The diastereomers (de = 4.7%) were not separated. Yield: 17%; IR: $\tilde{\nu} = 3003$, 2956, 2850, 1730, 1437, 1365, 1196, 1170; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.77$ (d, 1H, $^1CH_2$, $^3J_{1A,2} =$ 6.82 Hz), 2.18 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.54$ Hz), 2.43 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.54$, 6.82 Hz), 2.66–2.77 (m, 2H, ^3CH, $^4CH_2$), 2.95–3.02 (m, 1H, $^4CH_2$), 3.64, 3.67, 3.69 ppm (s, je 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 34.51$ ($^1CH_2$), 36.61 ($^4CH_2$), 37.05 (^2CH), 52.03, 52.43, 52.55 (OCH_3), 66.68 (^3CH), 170.19, 170.26, 171.19 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.96$ (d, 1H, $^1CH_2$, $^3J_{1A,2} =$ 6.82 Hz), 2.30 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.29$ Hz), 2.23 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.29$, 6.82 Hz), 2.66–2.77 (m, 2H, ^3CH, $^4CH_2$), 2.95–3.02 (m, 1H, $^4CH_2$), 3.64, 3.66, 3.70 ppm (s, je 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 33.52$ ($^1CH_2$), 36.78 ($^4CH_2$), 37.77 (^2CH), 52.03, 52.39,

52.52 (OCH_3), 66.96 (^3CH), 170.37, 170.49, 171.11 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{10}H_{15}NO_6$, 245.23; found: $[M+H]^+$ 246.2; LC-MS: $R_t = 1.2$ min, purity: >99%; $[\alpha]_D^{26} = +39$ ($CHCl_3$, $c = 0.1$).

Di-*tert*-butyl (*S*)-2-(2-methoxycarbonyl-aziridine-2-yl)-succinate (**18c**): The diastereomers (de = 14%) were not separated. Yield: 31%; IR: $\tilde{\nu} = 3004$, 2952, 2458, 1728, 1457, 1367, 1198, 1142, 766; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.37$–1.40 (m, 18H, $C(CH_3)_3$), 1.78 (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.83$ Hz), 2.14 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.54$ Hz), 2.23–2.26 (m, 1H, ^2CH), 2.51–2.59 (m, 2H, ^3CH, $^4CH_2$), 2.82–2.89 (m, 1H, $^4CH_2$), 3.67 ppm (s, 3H, O-CH_3); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 27.90$, 27.99, 28.03 ((CH_3)$_3$), 34.26 ($^1CH_2$), 37.05 (^2CH), 37.98 ($^4CH_2$), 52.26 (OCH_3), 67.72 (^3CH), 81.14, 82.04 (C_q), 168.90, 170.07, 170.71 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.37$–1.40 (m, 18H, $C(CH_3)_3$), 1.96 (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.56$ Hz), 2.23–2.26 (m, 2H, $^1CH_2$), 2.40 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.54$, 6.56 Hz), 2.51–2.59 (m, 2H, ^3CH, $^4CH_2$), 2.82–2.89 (m, 1H, $^4CH_2$), 3.65 ppm (s, 3H, O-CH_3); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 27.90$, 27.99, 28.03 ((CH_3)$_3$), 33.61 ($^1CH_2$), 37.81 (^2CH), 38.12 ($^4CH_2$), 52.26 (OCH_3), 67.81 (^3CH), 81.14, 82.04 (C_q), 169.20, 170.26, 170.66 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{16}H_{27}NO_6$, 329.40; found: $[M+H]^+$ 330.3; LC-MS: $R_t = 1.2$ min, purity 96.5%; $[\alpha]_D^{26} = +51$ ($CHCl_3$, $c = 0.1$).

Methyl (*S*)-2-({1-[(methoxycarbonylmethyl-carbamoyl)-methyl]-aziridine-2-carbonyl}-amino)-3-phenyl-propionate (**19**) was synthesized starting from methyl-2[(2,3-dibromopropanoyl)amino]-3-phenylpropanoate with Gly-GlyOMe.[15] The diastereomers (1/1) were not separated. Yield: 9%; IR: $\tilde{\nu} = 3061$, 3029, 2962, 2845, 1741, 1436, 1369, 1206, 1179, 758, 700; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.52$ (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.57$ Hz), 1.74 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.54$ Hz), 1.98–2.02 (m, 1H, ^2CH), 2.87–3.10 (m, 6H, $^3CH_2$, $^6CH_2$, $^5CH_2$),3.61, 3.62 (OCH_3), 4.68–4.78 (m, 1H, ^4CH), 6.98–7.21 ppm (m, 5H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 34.27$ ($^1CH_2$), 37.99 ($^5CH_2$), 39.74 (^2CH), 51.26, 52.36 (OCH_3), 53.22 (^4CH), 60.26 ($^3CH_2$), 125.92 ,127.41, 129.32 (CH, arom.), 135.98 (C_q, arom.), 169.32, 171.22 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.53$ (d, 1H, $^1CH_2$, $^3J_{1A,2} = 7.08$ Hz), 1.73 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.28$ Hz), 1.98–2.02 (m, 1H, ^2CH), 2.87–3.10 (m, 6H, $^3CH_2$, $^6CH_2$, $^5CH_2$), 3.57, 3.59 (OCH_3), 4.68–4.78 (m, 1H, ^4CH), 6.98–7.21 ppm (m, 5H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 36.48$ ($^1CH_2$), 38.13 ($^5CH_2$), 39.81 (^2CH), 51.47, 52.31 (OCH_3), 54.32 (^4CH), 60.55 ($^3CH_2$), 126.40, 127.67, 129.26 (CH, arom.), 135.98 (C_q, arom.), 169.36, 170.92 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{18}H_{23}N_3O_6$, 377.40; found: $[M+H]^+$ 378.4; LC-MS: $R_t = 1.4$ min, purity: 90.7%; $[\alpha]_D^{26} = -5$ ($CHCl_3$, $c = 0.1$).

20 and **21** were synthesized by reaction of benzylamine with methyl 2-[(2-bromo-acryloyl)amino]-3-methylbutanoate (**20**) or methyl 2[(2,3-dibromopropanoyl)amino]-3-phenylpropanoate (**21**).[15]

Methyl (*S*)-2-[(1-benzyl-aziridine-2-carbonyl)-amino]-3-methyl-butanoate (**20**): The diastereomers (de = 20%) were not separated. Yield: 23%; IR: $\tilde{\nu} = 3063$, 3030, 2963, 2876, 1739, 1452, 1355, 1206, 1151, 734, 698; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 0.75$–0.92 (m, 6H, $^6CH_3$), 1.73 (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.82$ Hz), 1.92 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 2.53$ Hz), 2.02–2.11 (m, 1H, ^5CH), 2.06–2.09 (m, 1H, ^2CH), 3.21 (s, 1H, ^3CH), 3.65 (s, 3H, OCH_3), 4.35–4.41 (m, 1H, ^4CH), 6.89 (bs, 1H, NH), 7.19–7.28 ppm (m, 5H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 17.50$ ($^6CH_3$), 18.95 ($^6CH_3$), 30.93 (^5CH), 35.15 ($^1CH_2$), 38.98 (^2CH), 52.04 (OCH_3), 56.35 (^4CH), 62.87 ($^3CH_2$), 127.46, 127.93, 128.50 (CH, arom.), 136.26, 136.90 (C_q, arom.), 169.91, 171.53 ppm (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 0.75$–0.92 (m, 6H, $^6CH_3$), 1.74 (d, 1H, $^1CH_2$, $^3J_{1A,2} = 7.07$ Hz), 2.02 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.03$ Hz), 2.02–2.11 (m, 1H, ^5CH), 2.13 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.03$, 7.07 Hz), 3.24 (s, 1H, ^3CH), 3.63 (s, 3H, OCH_3), 4.35–4.41 (m, 1H, ^4CH), 6.99 (bs, 1H, NH), 7.19–7.28 ppm (m, 5H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 17.69$ ($^5CH_3$), 19.01 ($^6CH_3$), 31.27 (^5CH), 35.48 ($^1CH_2$), 39.12 (^2CH), 52.08 (OCH_3), 56.31 (^4CH), 62.87 ($^3CH_2$), 127.46, 127.93, 128.50 (CH, arom.), 136.26, 136.90 (C_q, arom.), 169.91, 171.53 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{16}H_{22}N_2O_3$, 290.37; found: $[M+H]^+$ 291.4; LC-MS: $R_t = 3.7$ min, purity: 91.2%; $[\alpha]_D^{26} = +5$ ($CHCl_3$, $c = 0.1$).

Methyl (*S*)-2-[(1-benzyl-aziridine-2-carbonyl)-amino]-3-phenyl-propionate (**21**): The diastereomers (de = 4.7%) were not separated. Yield: 16%; IR: $\tilde{\nu} = 3061$, 3029, 3005, 2951, 2845, 1741, 1452, 1349, 1202, 1177, 740, 698; diastereomer 1: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.60$ (d, 1H, $^1CH_2$, $^3J_{1A,2} = 7.07$ Hz), 1.65 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.03$ Hz), 2.11 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.03$, 7.07 Hz), 2.91–3.10 (m, 2H, $^3CH_2$), 3.25–3.67 (m, 2H, $^5CH_2$), 3.65 (s, 3H, OCH_3), 4.70–4.76 (m, 1H, ^4CH), 6.90 (bs, 1H, NH), 7.17–7.35 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 35.14$ ($^1CH_2$), 37.93 ($^5CH_2$), 38.88 (^2CH), 52.28 (OCH_3), 52.39 (^4CH), 62.79 ($^3CH_2$), 127.10, 127.48, 127.86, 128.03, 128.45, 128.51, 129.18 ppm (CH, arom.), 135.86, 137.88 (C_q, arom.), 170.11, 171.73 (C=O); diastereomer 2: ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.68$ (d, 1H, $^1CH_2$, $^3J_{1A,2} = 6.83$ Hz), 1.93 (d, 1H, $^1CH_2$, $^3J_{1B,2} = 3.04$ Hz), 2.07 (dd, 1H, ^2CH, $^3J_{2,1A/B} = 3.04$, 6.83 Hz), 2.91–3.10 (m, 2H, $^3CH_2$), 3.25–3.67 (m, 2H, $^5CH_2$), 3.61 (s, 3H, OCH_3), 4.70–4.76 (m, 1H, ^4CH), 6.79 (bs, 1H, NH), 7.17–7.35 ppm (m, 10H, arom.); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 35.21$ ($^1CH_2$), 37.89 ($^5CH_2$), 38.88 (^2CH), 52.24 (OCH_3), 52.32 (^4CH), 62.79 ($^3CH_2$), 127.10, 127.48, 127.86, 128.03, 128.45, 128.51, 129.18 (CH, arom.), 135.86, 137.88 (C_q, arom.), 170.11, 171.73 ppm (C=O); LOOP-ESI-MS: calcd. f. $C_{20}H_{22}N_2O_3$, 338.41; found: $[M+H]^+$ 339.4; LC-MS: $R_t = 1.4$, 2.4 min, purity: 97.5%; $[\alpha]_D^{26} = +13$ ($CHCl_3$, $c = 0.1$).

3-Ethyloxycarbonyl-2-[(*N*-*tert*-butoxycarbonyl)piperazin-*N*-carbonyl] aziridine (**46**) was synthesized by DPPA (diphenyl phosphoryl azide) mediated peptide coupling[34] of aziridine-2,3-dicarboxylic acid monoethyl ester with Boc-piperazine. Yield: 66%; IR (neat):$\tilde{\nu}$ 3459, 1733, 1671, 1639, 1590, 1484, 1187, 1137, 924, 725, 689 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 4.29$–4.09 (m, OCH_2, 2H); 3.70–3.34 (br. m, 4xCH_2, 8H); 2.99 (br. s, 3-C*H* Azi, 1H); 2.70 (br. s, 2-C*H* Azi, 1H); 2.16 (br. s, N*H*); 1.44 (s, BOC, 9H); 1.27 ppm (t, $J = 7.1$ Hz, CH_3, 3H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 169.3$ (N-*C*=O); 166.9 (*C*=O(OEt)); 154.3 (*C*=O BOC); 80.4 (q. C); 61.7 (OCH_2CH_3); 44.8 (CH_2); 43.5 (CH_2); 43.2 (CH_2); 42.5 (CH_2); 35.6 (2-*C*H Azi); 33.7 (3-*C*H Azi); 28.3 (CH_3 BOC); 14.0 ppm (OCH_2CH_3); HR-ESI-MS: calcd. f. $C_{15}H_{25}N_3O_5$, $[M+H^+]$: 328.1872; found: 328.1866; $[\alpha]_D^{26} = +61.1$ ($c = 1.44$, $CHCl_3$).

Bis-*N,N*-(3-ethoxycarbonylaziridine-2-carbonyl) piperazine (**47**) was synthesized by DPPA mediated peptide coupling[34] of 2 equiv aziridine-2,3-dicarboxylic acid monoethyl ester with 1 equiv piperazine. Yield: 29%; IR (neat): $\tilde{\nu} = 3461$, 1734, 1640, 1591, 1485, 1446, 1256, 1188, 1138, 1022, 976, 925, 774, 726, 690 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 4.29$–4.16 (m, OCH_2CH_3, 4H); 3.61–3.22 (br. m, Prz CH_2, 8H); 2.97 (dd, $J = 2.0$ Hz, 8.3 Hz, Azi C*H*, 1H); 2.69 (dd, $J = 2.0$ Hz, 9.2 Hz, Azi C*H*, 1H); 2.17 (t, $J = 8.6$ Hz, Azi N*H*, 1H); 1.3 ppm (t, $J = 7.2$ Hz, OCH_2CH_3, 6H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 169.24$, 166.91 (N-*C*=O); 61.69 (*C*=O(OEt)); 45.25 (OCH_2CH_3); 44.8, 44.3, 42.8 (CH_2 Prz); 35.8, 33.7 (Azi *C*H); 14.1 ppm (OCH_2CH_3); HR-ESI-MS: calcd. f. $C_{16}H_{24}N_4O_6$, $[M+H^+]$: 369.1774; found: 369.1767; $[\alpha]_D^{25} = -38.6$ ($c = 1.05$, $CHCl_3$).

3-((*S*)-1-*tert*-Butoxycarbonyl-3-carbamoyl-propylcarbamoyl)-aziridine-2-carboxylic acid ethyl ester (**48**) was synthesized by DPPA mediated peptide coupling[34] of aziridine-2,3-dicarboxylic acid monoethyl ester with Gln*O*tert*Bu*. Yield: 47%; IR (neat): $\tilde{\nu} = 3410$, 3325, 2978, 2359, 1728, 1655, 1539, 1370, 1202, 1163, 1030, 991, 848, 811, 632 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 1.30$ (t, 3H, CH_2CH_3), 1.45 (s, 9H, *tert*-But), 1.80 (br s, 1H, 1-H), 1.84–1.95 (m, 1H, 6-H), 2.12–2.32 (m, 3H, 6-H and 7-H), 2.66 (d, 1H, $J = 2.0$ Hz, 2-H or 3-H), 2.82, (d, 1H, $J = 2.0$ Hz, 2-H or 3-H), 4.22 (dq, 2H, $J =$

2.5 Hz, $J=7.1$ Hz, CH_2CH_3), 4.38–4.66 (m, 1H, 5-H), 5.48 (br s, 1H, 8-NH), 6.15 (br s, 1H, 4′-NH), 7.15 ppm (d, 1H, $J=7.8$ Hz, 5-NH); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=14.10$, 27.96, 28.63, 31.90, 35.64, 37.48, 52.09, 62.23, 82.87, 168.4, 170.5, 174.3 ppm; ESI-MS calcd. for $C_{15}H_{25}N_3O_6$, 343.38; found: 709.3 [2M<M+>Na], 366.4 [M+Na], 344.2 [M+1]; $[\alpha]_D^{25}=60.5$ ($c=0.60$, MeOH).

3-{[(4-Methoxy-phenylcarbamoyl)-methyl]-carbamoyl}-aziridine-2-carboxylic acid ethyl ester (**49**) was synthesized by DPPA mediated peptide coupling[34] of aziridine-2,3-dicarboxylic acid monoethyl ester with glycine 4-methoxyphenyl amide. Yield: 27%; IR (neat): $\tilde{\nu}=3298$, 3102, 2974, 2954, 2928, 2837, 1742, 1719, 1665, 1645, 1515, 1372, 1246, 1217, 1183, 1028, 856, 817, 680 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=1.23$ (t, 3H, $J=7.1$ Hz, CH_2CH_3), 1.61 (s, 1H, 1-H), 2.64 (br s, 1H, 2-H or 3-H), 2.83 (br s, 1H, 2-H or 3-H), 3.72 (s, 3H, OCH_3), 3.97 (m, 2H, 5-H), 4.17 (dq, 2H, $J=7.1$ Hz, $J=1.7$ Hz, CH_2CH_3), 6.78 (d, 2H, $J=9.1$ Hz, 8-H), 7.14 (br s, 1H, NH), 7.33 (d, 2H, $J=9.1$ Hz, 9-H), 7.98 ppm (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=14.09$, 35.82, 37.38, 43.87, 55.48, 62.36, 114.2, 121.8, 130.4, 156.7, 166.3, 169.1 ppm; ESI-MS calcd. for $C_{15}H_{19}N_3O_5$, 321.4; found: 665[2M+Na], 643.3 [2M+1], 344.1 [M+Na], 322.1 [M+1]; $[\alpha]_D^{25}=75.8$ ($c=0.58$, MeOH).

3-((*S*)-1-*tert*-Butoxycarbonyl-2-carbamoyl-ethylcarbamoyl)-aziridine-2-carboxylic acid ethyl ester (50) was synthesized was synthesized by DPPA mediated peptide coupling[34] of aziridine-2,3-dicarboxylic acid monoethyl ester with AsnO*tert*Bu. Yield: 45%; IR (neat): $\tilde{\nu}=3406$, 3260, 2978, 2932, 1722, 1665, 1562, 1442, 1412, 1370, 1252, 1217, 1157, 847 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=$ 1.27 (t, 3H, $J=7.1$ Hz, CH_2H_3), 1.44 (s, 9H, CO_2*t*Bu), 2.65 (br. s, 1H, 2-H or 3-H), 2.72 (dd, 1H, $J=15.9$ Hz, $J=4.3$ Hz, 6-H), 2.84 (dd, 1H, $J=15.9$ Hz, $J=5.1$ Hz, 6-H), 2.87–2.82 (br. s, 1H, 2-H or 3-H), 4.26–4.18 (m, 2H, CH_2H_3), 4.61 (dt, 1H, $J=7.8$ Hz, $J=4.8$ Hz, 5-H), 5.78 (br. s, 1H, 7-NH), 5.94 (br. s, 1H, 7-NH), 7.45 ppm (d,1H, $J=7.8$ Hz, 5-NH); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=14.08$, 27.86, 37.07, 49.40, 62.12, 82.76, 170.0, 172.1 ppm; ESI-MS calcd. for $C_{14}H_{23}N_3O_6$, 329.35; found: 681.3 [2M+Na], 659.3 [2M+1], 352.2 [M+Na], 330.2 [M+1]; $[\alpha]_D^{25}=61.4$ ($c=0.55$, MeOH).

Biological evaluations

Protease inhibition

Enzyme assays with cysteine proteases were performed with Cbz-Phe-Arg-AMC (falcipain-2) and Cbz-Leu-Arg-AMC (falcipain-3) as substrates (Calbiochem or Bachem) (concentrations: 25 μM for recombinant falcipain-3; 50 μM for recombinant falcipain-2) according to previously described assays.[21–23,25] The enzymes were incubated with different concentrations of the compounds for 0, 15, or 30 min prior to substrate addition. Compound concentrations ranged from those that minimally inhibited to those that fully inhibited the enzyme. Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed in 96-well plates in a total volume of 120 or 300 μL ($n=2$–6 independent assays). The standard deviations are <10% in all cases. The following buffer was used: 100 mM sodium acetate, pH 5.5, 10 mM DTT for falcipain-2 and -3. For the determination of IC_{50} values the increase of fluorescence over the time was monitored continuously at room temperature for 15 min. For the determination of K_i, k_i and k_{2nd} values fluorescence was monitored up to 45 min. Either a Labsystems Fluoroscan II spectrofluorometer, a TECAN Spectra Fluor microplate reader (TECAN, Deutschland GmbH, Crailsheim, Germany) or a Varian Cary Eclipse spectrofluorometer Varian, Darmstadt, Germany with a microplate reader (excitation 365 nm, emission 460 nm, in all cases) was used. IC_{50} values were determined from plots of percent activity over compound concentration using GraphPad Prism software or Grafit software.[35] For determination of K_i, k_i and k_{2nd} values for falcipain-2 the inactivation rates (k_{obs}) for different inhibitor concentrations in the presence of the substrate (25 μM, 40 μM, or 80 μM) were determined according to the continuous method of Tian and Tsou[24] by monitoring the product released from hydrolysis of the substrate in presence of the inhibitor as a function of time (fluorescence $=A(1-\exp(-k_{obs}t))+B$). Fitting of the k_{obs} values against the inhibitor concentrations to the hyperbolic equation $k_{obs}=k_i$ [I]/K_i^{app} + [I] gave the individual values of K_i^{app} and k_i. The K_i^{app} values were corrected to zero substrate concentration by the term $(1+[S]/K_m)$ in equation $K_i=K_i^{app}/(1+[S]/K_m)$. The second-order rate constants $k_{2nd}=k_i/K_i$ were directly calculated from the individual constants. K_i and k_i values were calculated by nonlinear regression analyses using the program GraFit.[35] The K_m value for falcipain-2 used to correct K_i^{app} values was 21.5 μM.

Evaluations of cultured malaria parasites

Intraerythrocytic parasites (strain W2, and strain FCBR, the latter kindly provided by K. Lingelbach, Philipps-University Marburg, Germany; for information about strain FCBR see: http://pathport.vbi.vt.edu/pathinfo/pathogens/falciparum.html) were grown in vitro in human RBCs (red blood cells, blood group A^{Rh+}) under standard conditions[36] using RPMI 1640 medium (Sigma Aldrich) supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, and 0.5% AlbuMAX I (Invitrogen) at 2 to 5% (*v*/*v*) hematocrit. Beginning at the ring stage the compounds were tested for their ability to inhibit parasites by incubating with different concentrations. For the preliminary screening concentrations of 1, 10, and 100 μM inhibitor were used. After 48 h the Albumax medium was removed and cultures were incubated with 1% formaldehyde in PBS (pH 7.4) for about 2 days. The fixed parasites were transferred into 0.1% Triton-X-100 in PBS containing 1 nM YOYO-1 dye. The parasites were counted by fluorescence-activated cell sorting (FACS) analysis, and counts were compared with those of untreated controls. Inhibitors were dissolved in DMSO. The maximum concentration of DMSO used in this assay was 0.1%. Two independent assays were performed in 200 μL final volume in 96-well plates. The standard deviations are <10% in all cases. IC_{50} values were determined by linear regression analyses on the linear segments of the dose-response curves. In parallel, viability assays were performed using the microculture tetrazolium test (MTT), or by the assessment of parasite development using Hoechst-33258 as DNA stain according to refs. [29, 30].

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