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Michael Acceptor Based Antiplasmodial and Antitrypanosomal Cysteine Protease Inhibitors with Unusual Amino Acids

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New peptidic Michael acceptor based cysteine protease inhibitors displaying antiparasitic activity were identified by testing a broad series of 45 compounds in total, containing Asn, Gln, or Phe. As target enzymes, falcipain-2 and -3 from *P. falciparum* and rhodesain from *T. b. rhodesiense* were used. In the case of the Asn/Gln containing compounds, the trityl-protected, diastereomeric *E*-configured vinylogous dipeptide esters **16** (Boc-(*S*)-Phg-(*R*/*S*)-vGln(Trt)-OEt) were discovered as most active inhibitors concerning both protease inhibition and antiparasitic acitivity, with inhibition constants in the submicromolar range. The compounds were shown to display time-dependent and competitive inhibition. In the case of the Phe containing compounds, the maleic acid derivatives **42** and **43** (BnO-Phe←Mal-Phe-OBn, BnO-Phe←Mal-Phe-Ala-OBn, Mal = maleic acid) displayed good inhibition of rhodesain as well as good antitrypanosomal activity, while the fumaric acid derived *E*-analogue **14** (BnO-Phe←Fum-Phe-OBn) only displayed inhibition of the target enzymes but no antiparasitic activity. Inhibition by these Phe derivatives was shown to be time-independent and competitive.

Introduction

The burden of tropical diseases of humans caused by protozoan parasites is large, in terms of mortality and morbidity, and because these diseases impede economic growth and prosperity.[1] Diseases such as malaria (caused by various species of *Plasmodium*) and African trypanosomiasis (sleeping sickness caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*) are among the most severe.[2,3] Late-stage trypanosomiasis is characterized by somnolence and coma, leading invariably to death if untreated. Chemotherapy depends principally on drugs developed decades ago that lack adequate efficacy and cause serious side effects. Further, the emergence of drug-resistant *Trypanosoma* strains has been reported.[2] Similarly in malaria, increasing resistance of malaria parasites to antimalarial drugs, the lack of highly effective vaccines, and inadequate control of mosquito vectors demand new approaches to drug development.[4−7]

In both diseases one promising strategy to develop new drugs has been to target parasite cysteine proteases.[3,8] These enzymes, termed rhodesain[9] (RD[a]) in *T. b. rhodesiense*, brucipain in *T. b. brucei* (infective to animals), and falcipains[10−13] (falcipain-2, falcipain-2′, and falcipain-3) in *Plasmodium falciparum*, belong to the cathepsin L subfamily of the papain family (clan CA, family C1; CAC1)[14] of cysteine proteases. Cysteine protease inhibitors have been shown to kill African trypanosomes in vitro and in animal models of the disease,[3] although it is not yet clear whether rhodesain is the only target of the inhibitors.[15] Proteases of malaria parasites play pivotal roles in the processes of host erythrocyte rupture, erythrocyte invasion, and hemoglobin degradation. Treatment with cysteine protease inhibitors blocks hemoglobin hydrolysis and development of the parasite.[6,16−19] Among the falcipains, falcipain-2 and falcipain-3 are likely the major hemoglobinases in the food vacuole of erythrocytic parasites.[8,12,20] Therefore, the inhibition of cysteine proteases presents a promising strategy for combating these infections.

In a recent paper we described the synthesis of a combinatorial heptapeptide library (Figure 1) based on fumaric acid as an electrophilic building block.[21] The selection of amino acids

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[a] Abbreviations: ACN, acetonitrile; C1-NHAc, acetylaminoalanine; cc, column chromatography; Chg, cyclohexylglycine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIBAL-H, diisobutylaluminum hydride; DIPEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DPPA, diphenylphosphorylazide; DTT, dithiothreitole; FP-2, falcipain-2; FP-3, falcipain-3; Fum, fumaric acid; HCTU, 2-(6-chloro1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; IBCF, isobutyl chloroformate; Mal, maleic acid; NBS, *N*-bromosuccinimide; NMM, *N*-methylmorpholine; Phg, phenylglycine; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; RD, rhodesain; rt, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Trt, trityl; vGln, vinyl-Gln. Amino acids are (*S*)-configured unless otherwise indicated.

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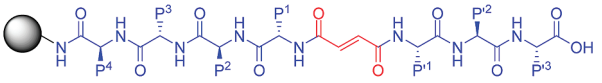
for the library considered the known preference of CAC1 proteases for hydrophobic amino acids in the P2[22] position (Leu, Ile, Phe, cyclohexylglycine Chg, phenylglycine Phg). For P3, different unpolar amino acids were used (Val, Ala, Leu), whereas P4 amino acids were held constant and those at P1′ only marginally varied (Gly, Ala). For the P1-position either nonpolar amino acids (Phe, Ala) or amino acids with amide side chains (Gln, Asn, C1-NHAc) were chosen. Via a new high-throughput on-bead assay, inhibitors with amino acids unusual for cathepsin-L like cysteine proteases were identified,[21] namely, Gln and Asn at P1 and Phg and Chg at P2.

We now present the synthesis and testing of a large series of related peptidic inhibitors containing these amino acids or respective peptides but with various modified Michael systems, e.g., trans- and cis-configured vinylogous amino acid or peptide esters, crotonic acid derivatives, and acrylic acid derivatives. Notably, these studies revealed trityl-protected Asn- and Gln-derivatives as highly potent inhibitors.

The manuscript is organized as follows: first, we describe the syntheses of the potential inhibitors. Second, the biological activities against the target enzymes (falcipain-2, falcipain-3, rhodesain) and the parasites (*P. falciparum*, *T. b. brucei*) and cytotoxicity data are presented. A chapter about the clarification of enzyme inhibition mechanisms and the discussion of the structure−activity relationships (SAR) complete the manuscript.



Cpd.	P4[a]	P3[a]	P2[a]	P1[a]	P1'[a]	P2'[a]	P3'[a]
Amino acid residues used in subsite mapping	Val	Val	Leu	Gln	Gly	Gly	Phe
		Ala	Ile	Asn	Ala		
		Leu	Phe	C1-NHAc[b]			
			Chg	Ala			
			Phg	Phe			
Lead inhibitors							
1	Val	Leu	Leu	Phe	Gly	Gly	Phe
2	Val	Val	Chg	Asn	Gly	Gly	Phe
3	Val	Val	Chg	Gln	Gly	Gly	Phe

Figure 1. Composition of the heptapeptide fumaric acid diamide library: structures of inhibitors **1**−**3**.[21]. (a) Because of the insertion of the fumaric acid moiety into the peptide, the definition of the amino acid residues as P1, P2, P1′, P2′, etc. does not implicate binding of these residues into the respective substrate binding pockets (S1, S2, S1′, S2′, etc.) of the enzyme. Depending on the carbon atom attacked by the nucleophilic cysteine residue of the enzyme, the binding mode may be shifted to either the P- or P′-direction.[21] (b) C1-NHAc, acetylaminoalanine.

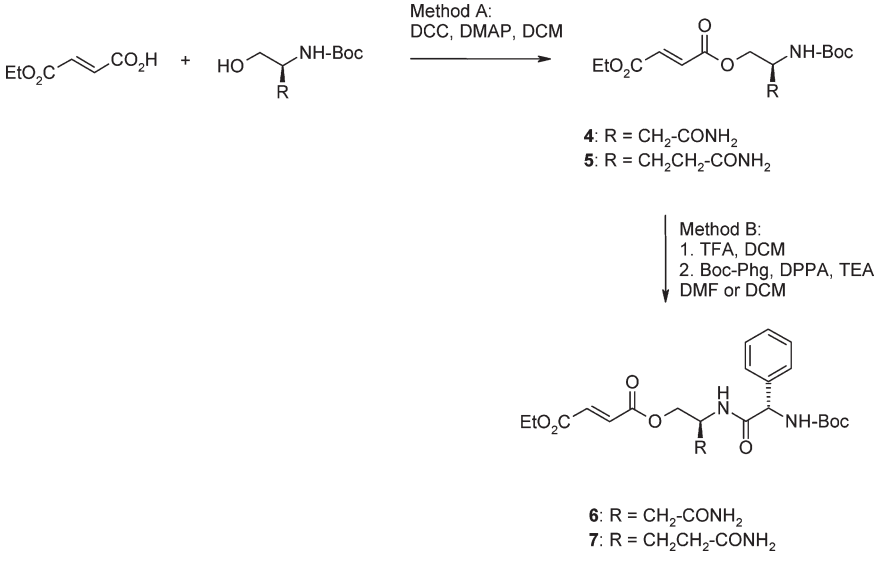
Results

Since the recently described studies[21] yielded inhibitor H_2N-Val←Leu←Leu←Phe←Fum-Gly-Gly-Phe-OH (**1**) containing Phe in the P1-position and inhibitors H_2N-Val←Val←Chg←Asn←Fum-Gly-Gly-Phe-OH (**2**) and H_2N-Val←Val←Chg←Gln←Fum-Gly-Gly-Phe-OH (**3**) containing Asn or Gln in the P1-position as the most potent inhibitors (Figure 1) (K_i values in μM for falcipain-2/rhodesain: (**1**) 0.8/0.2; (**2**) 5.2/0.08; (**3**) 7.0/0.3), we now prepared compounds with the same amino acids but with a modified Michael system. The various Michael acceptors, which are bound to Gln, Asn, and Phe, or di-, tri- and tetrapeptide sequences of the nonprimed site of the fumaric acid library (Phg/Chg/Leu-Asn/Gln; Ala-Leu-Gln; Ala-Val-Chg-Gln; Val-Val-Leu-Gln) were synthesized as depicted in Schemes 1−10. Since the syntheses of the respective peptides yielded several trityl-protected peptides (Trt = trityl) as intermediates, these were also coupled to the various Michael acceptors, and the resulting inhibitors were included for testing. In addition, for selected compounds, the analogues with *Z*-configured activated double bonds were synthesized and tested (Schemes 11 − 13). Inhibitor structures and results of biological tests are summarized in Table 1.

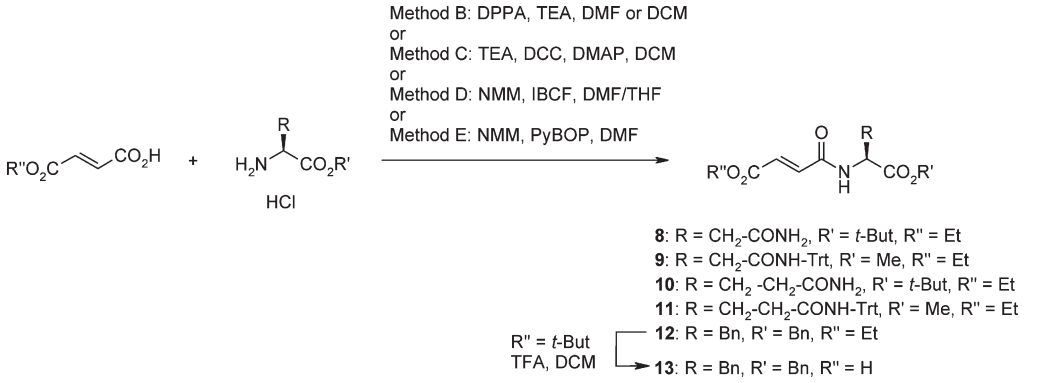
Syntheses. Compounds **4** and **5** were synthesized by Steglich esterification of fumaric acid monoethyl ester with Boc-asparaginol or Boc-glutaminol (Scheme 1). Deprotection with TFA and DPPA-mediated peptide coupling with Boc-Phg yielded inhibitors **6** and **7**.

The fumaric acid amides **8**−**12** (Scheme 2) were synthesized starting from fumaric acid monoethyl ester and Asn-O-*t*-But (→**8**), Trt-protected Asn-OMe (→**9**), Gln-O-*t*-But (→**10**), Trt-protected Gln-OMe (→**11**), or Phe-OBn (→**12**) by various standard peptide coupling methods. For the synthesis of the free acid **13** fumaric acid mono-*tert*-butyl ester[21] was used. Final cleavage of the *tert*-butyl ester with TFA in dichloromethane yielded compound **13**.

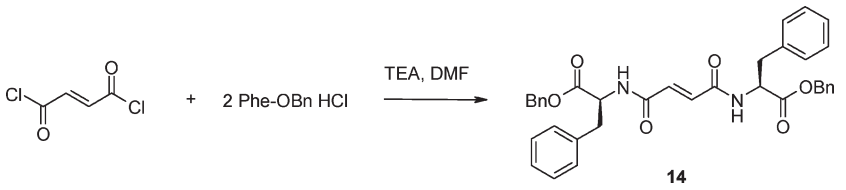
Scheme 1. Syntheses of Fumaric Acid Diester Based Inhibitors **4**−**7**



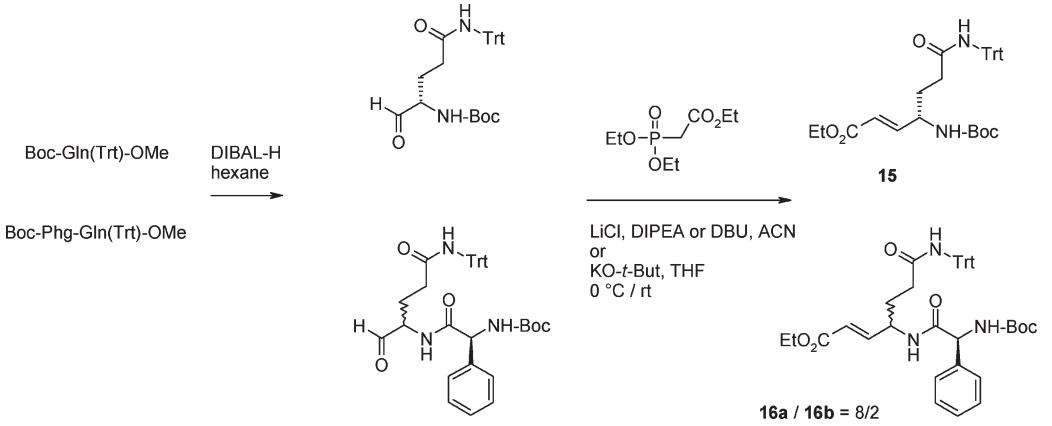
Scheme 2. Syntheses of the Fumaric Acid Amides **8**−**13**



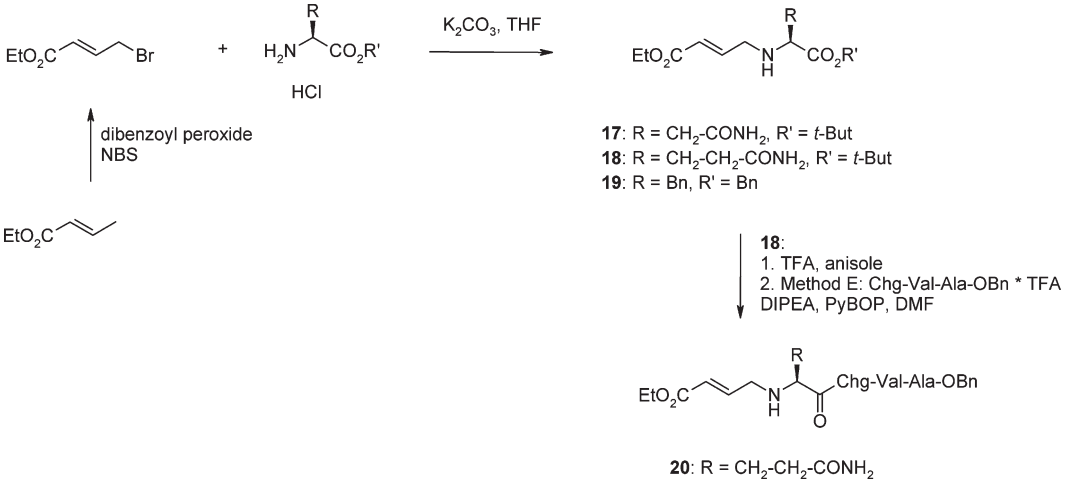
Scheme 3. Synthesis of the Fumaric Acid Diamide **14**



Scheme 4. Synthesis of the Vinylogous Amino Acid (**15**) and Dipeptide Esters (**16**)



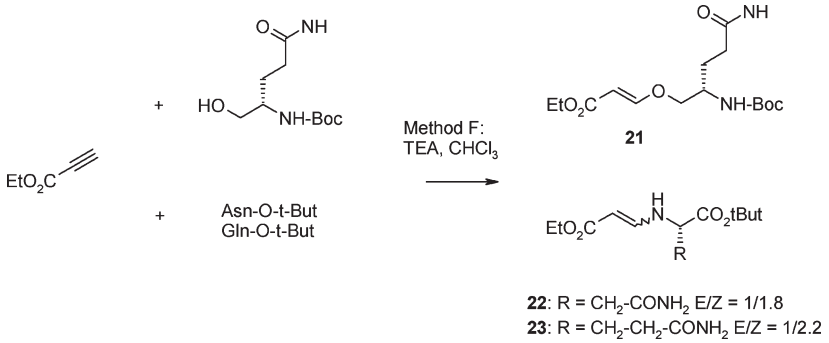
Scheme 5. Synthesis of Aminocrotonic Acid Esters **17**−**20**



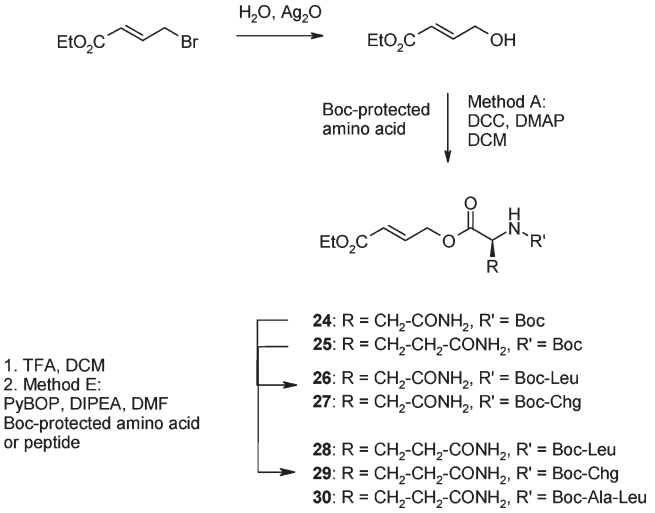
The fumaric acid diamide **14** was synthesized by reaction of fumaric acid dichloride with 2 equiv of Phe-OBn (Scheme 3).

The vinylogous amino acid ethyl esters **15** and **16** were obtained by Masamune reaction or Horner−Wadsworth−Emmons olefination (Scheme 4) of the aminoaldehyde

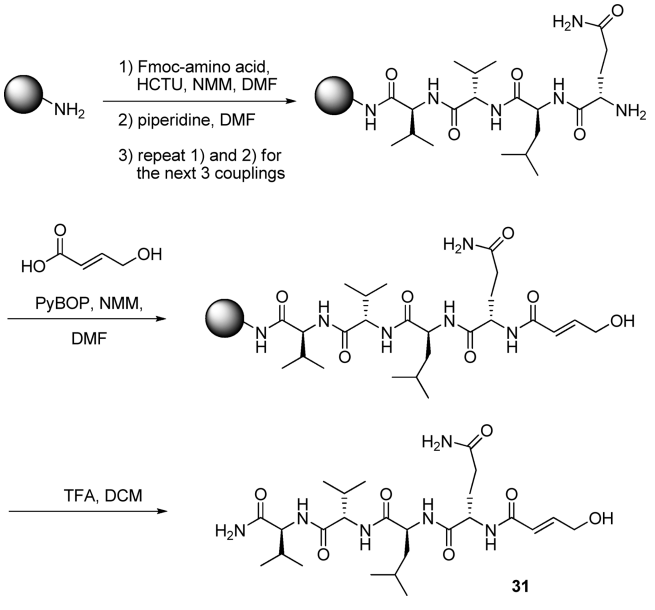
Scheme 6. Synthesis of Hydroxy- or Aminoacrylic Acid Esters **21**−**23**



Scheme 7. Synthesis of Hydroxycrotonic Acid Esters **24**−**30**

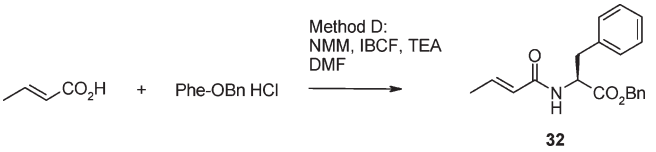


Scheme 8. Synthesis of the Hydroxycrotonic Acid Amide **31**

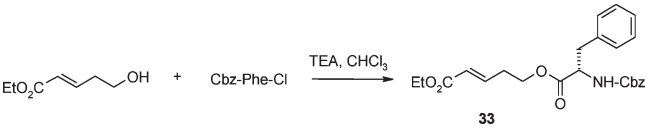


Boc-Gln(Trt)-H (→**15**) or the peptidylaldehyde Boc-Phg-Gln-(Trt)-H (→**16**) at room temperature. At this temperature only the respective *E*-isomers were obtained. In the case of the dipeptide **16** the DIBAL-H reduction of the methyl ester led to partial epimerization at the α-carbon atom of Gln(Trt). Thus, the subsequent olefination yielded two diasteromers **16a** and

Scheme 9. Synthesis of Crotonic Acid Amide **32**



Scheme 10. Synthesis of the Phe Ester **33**



16b in a ratio of 8:2, providing us with a very small amount of the minor isomer **16b** after chromatographic separation. Thus, this compound was at first only tested against the parasites. Since these assays showed **16b** to display very good antiparasitic activity, we resynthesized this diastereomer using (*R*)-Gln(Trt) instead of (*S*)-Gln(Trt).

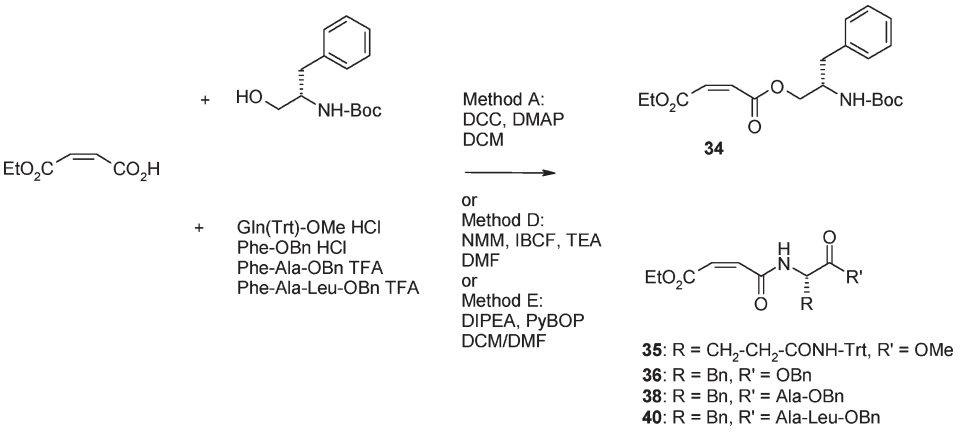
The aminocrotonic acid esters **17**−**19** (Scheme 5) were synthesized by N-alkylation of the respective amino acid esters Asn-O-*t*-But (→**17**), Gln-O-*t*-But (→**18**), or Phe-OBn (→**19**) with ethyl 4-bromocrotonate. Deprotection of the *tert*-butyl ester **18** with TFA/anisole and PyBOP-mediated peptide coupling with the tripeptide Chg-Val-Ala-OBn yielded compound **20**.

The Michael-type addition (Scheme 6) of either Boc-glutaminol (→**21**) or Asn- or Gln-O-*t*-But (→**22**, **23**) to ethyl propiolate yielded the hydroxyacrylic acid ester (**21**) and the aminoacrylic acid esters **22** and **23**. While the addition of the amino alcohol exclusively led to the *E*-isomer, the addition of the amino acids yielded the *E*- and *Z*-isomers in a ratio of about 1:2. Since no inhibition could be observed with the *E*/*Z*-mixture in preliminary studies, we did not separate the diastereomers.

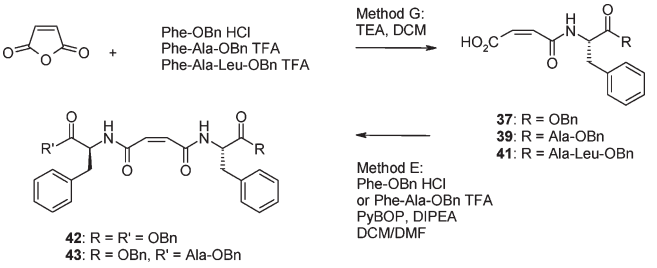
The hydroxycrotonic acid esters **24** and **25** (Scheme 7) were obtained by Steglich esterification of 4-hydroxycrotonic acid with the respective Boc-protected amino acids. Compounds **26**−**30** (Scheme 7) were obtained by deprotection of **24** and **25**, respectively, and PyBOP mediated peptide coupling with amino acids or peptides.

Compound **31** (Scheme 8) was synthesized on solid phase by using amino functionalized Sieber amide resin[23] and Fmoc-protected amino acids. The amino acids were activated with PyBOP in DMF and NMM as a base. An excess of NMM and 2.5 equiv of the amino acids and PyBOP were used in each coupling step. The 4-hydroxycrotonic acid was synthesized starting from crotonic acid based on published

Scheme 11. Synthesis of Maleic Acid Esters and Amides **34**−**36**, **38**, and **40**



Scheme 12. Synthesis of Maleic Acids **37**, **39**, **41** and Diamides **42** and **43**



procedures[24−27] and coupled after activation with PyBOP in DMF and NMM. Only 1.25 equiv of the reagents were used this time to prevent the reaction of the unprotected alcohol with the active ester. The crotonic acid amide **31** was obtained after cleavage from the resin with 1% TFA and purification by MPLC (RP-18 column, water and methanol).

Amide coupling (Scheme 9) of crotonic acid with Phe-OBn using isobutyl chloroformate as coupling reagent yielded the amide **32**.

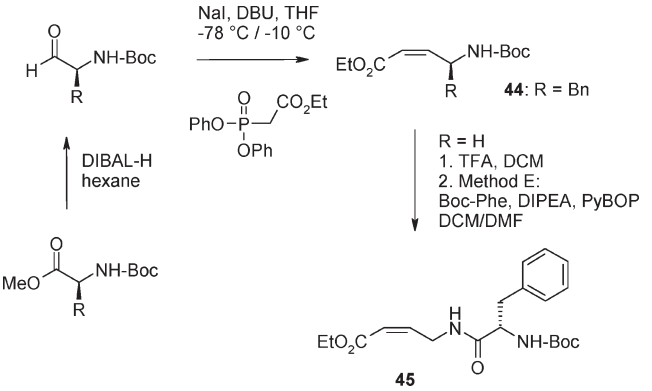
(*E*)-5-Hydroxypent-2-enoic acid ethyl ester, which was synthesized by Wittig reaction of 3-hydroxypropanal (obtained from reaction of acrolein with dilute sulfuric acid) with carboethoxymethyltriphenylphosphonium bromide, was reacted with the acid chloride of Cbz-protected Phe to yield the ester **33** (Scheme 10).

The maleic acid diester **34** and the amides **35**, **36**, **38**, and **40** (Scheme 11) were obtained either by Steglich esterification of maleic acid monoethyl ester with Boc-phenylalaninol (→**34**) or by various peptide coupling procedures with amino acid or peptide esters.

Acylation of amino acid or peptide esters with maleic acid anhydride yielded the maleic acids **37**, **39**, and **41**. The acid **37** was coupled via the PyBOP method to Phe-OBn or Phe-Ala-OBn in order to obtain the diamides **42** and **43** (Scheme 12).

Finally, the vinylogous amino acid ester **44** was obtained with high *Z*-selectivity (*Z*/*E* = 13/1) by Horner−Wadsworth−Emmons olefination (Scheme 13) of Boc-phenylalaninal at low temperature. The olefination of Boc-glycinal yielded the intermediate *Z*-configured vinylogous amino acid ester (R = H, *Z*/*E* = 2.5/1), which (after column chromatography to separate the *Z*- from the *E*-isomer) was then deprotected with TFA and coupled to Boc-Phe using PyBOP as coupling reagent to give inhibitor **45**.

Scheme 13. Syntheis of the Vinylogous *Z*-Configured Amino Acid Derivatives **44** and **45**



Biological Activities. The inhibitors were tested against falcipain-2 (FP-2), falcipain-3 (FP-3), and rhodesain (RD) in standard fluorescence assays as published previously.[28,29] The hydrolyses of Cbz-Phe-Arg-AMC (for RD) or Cbz-Leu-Arg-AMC (for FP-2 and FP-3) in the absence or presence of the respective inhibitor were measured by following the fluorescence increase due to release of AMC. The well-known cysteine protease inhibitor E-64,[30−32] as a positive control, and the solvent DMSO, as negative control, were used. Studies to clarify the inhibition mechanisms (time-dependent vs time-independent inhibition, competitive vs noncompetitive inhibition, effects of the low-molecular weight sulfur nucleophile DTT) as well as studies to exclude nonspecific inhibition by aggregation[33,34] were performed with the most active compounds. Inhibitors were further tested for antiplasmodial and antitrypanosomal activities. Antiplasmodial activities were determined by flow cytometry according to a previously published method using *P. falciparum* strain W2[12] or with the *P. falciparum* strain FCBR using a previously published assay.[35] Chloroquine[36] and E-64[37] were used as positive controls, and the solvent DMSO was used as a negative control. Antitrypanosomal activities were determined against *T. brucei brucei* as published earlier.[29] The well-known drugs melarsoprol, eflornithine, suramine, nifurtimox, and pentamidine were used as positive controls. All inhibition data are summarized in Table 1. For several compounds assays on mammalian macrophages (J774.1) to evaluate nonspecific toxicity were performed as previously described (Table 2).[29]

Studies on Inhibition Mechanisms. With some of the most active compounds (namely, inhibitors **14**, **15**, **16a**, **16b**, **20**,

Table 1. Inhibition of Falcipains (FP) and Rhodesain (RD) and Antiparasitic Activity of Michael Acceptors Containing Phe, Gln, or Asn[d]

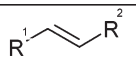
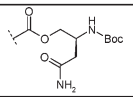
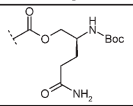
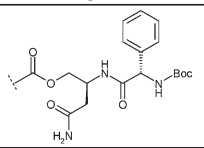
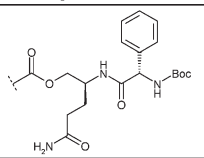
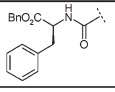
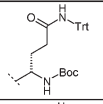
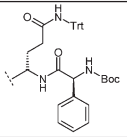
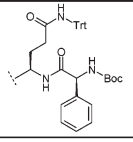
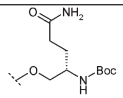
Inhibitors with *E*-configuration of the activated double bond 								
Fumaric acid diesters								
Comp.	**R^1**	**R^2**	**Config. double bond**	**FP-2 K_i [μM]**	**FP-3 K_i [μM]**	**RD K_i [μM]**	***P. falc.* W2 IC_{50} [μM]**	***T. b. b.* IC_{50} [μM]**
4	EtO_2C		*E*	n.i.	n.i.	n.i.	n.i.	n.i.
5	EtO_2C		*E*	n.i.	n.i.	n.i.	n.i.	n.i.
6	EtO_2C		*E*	45% (100 μM)	n.d.	51% (100 μM)	21.7[a]	33.2
7	EtO_2C		*E*	n.i.	n.d.	25% (100 μM)	n.d.	31.3
Fumaric acid amides								
8	EtO_2C	-CO-Asn-O-*t*-But	*E*	n.i.	n.i.	n.i.	n.i.	n.i.
9	EtO_2C	-CO-Asn(Trt)-OMe	*E*	12.0	n.i.	20% (100 μM)	7.3	25.1
10	EtO_2C	-CO-Gln-O-*t*-But	*E*	n.i.	n.i.	n.i.	75.3	n.i.
11	EtO_2C	-CO-Gln(Trt)-OMe	*E*	3.3	14.7	1.4	4.7	31.0
12	EtO_2C	-CO-Phe-OBn	*E*	4.7	65.9	7.6	n.i.	0.25
13	HO_2C	-CO-Phe-OBn	*E*	n.i.	n.d.	11% (100 μM)	n.i.	n.i.
14		-CO-Phe-OBn	*E*	0.98	2.8	0.34	n.i.	n.i.
Vinylogous amino acid esters								
15	EtO_2C		*E*	1.8	8.2	4.3	4.0	2.8
16a	EtO_2C		*E*	0.45	1.4	0.47	3.9	31.0
16b	EtO_2C		*E*	0.49	n.d.	0.14 ± 0.05	0.9[a]	3.5
Amino crotonic acid esters								
17	EtO_2C	-CH_2-Asn-O-*t*-But	*E*	n.i.	n.i.	n.i.	n.i.	n.i.
18	EtO_2C	-CH_2-Gln-O-*t*-But	*E*	n.i.	n.i.	n.i.	n.i.	n.i.
19	EtO_2C	-CH_2-Phe-OBn	*E*	152	n.i.	n.i.	n.i.	25.0
20	EtO_2C	-CH_2-Gln-Chg-Val-Ala-OBn	*E*	6.7	28.4	5.8	35.8	n.d.
Hydroxy acrylic acid esters								
21	EtO_2C		*E*	n.i.	n.i.	n.i.	n.i.	n.i.
Amino acrylic acid esters								
22	EtO_2C	Asn-O-*t*-But, *E*/*Z* = 1/1.8	*E*	n.i.	n.i.	n.i.	n.i.	n.i.
23	EtO_2C	Gln-O-*t*-But, *E*/*Z* = 1/2.2	*E*	n.i.	n.i.	n.i.	n.i.	n.i.

Table 1. Continued

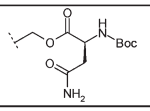
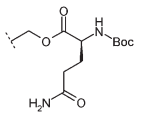
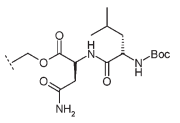
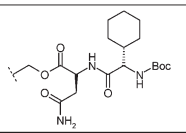
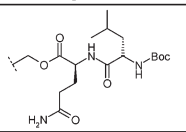
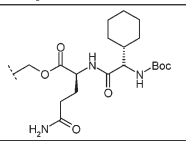
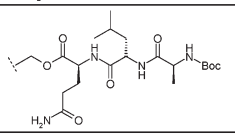
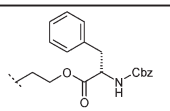
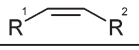
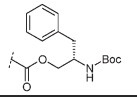
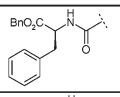
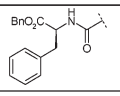
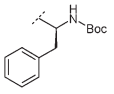
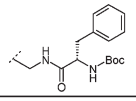
Hydroxy crotonic acid esters								
24	EtO_2C		*E*	17.0	58.0	n.i.	34.8	n.i.
25	EtO_2C		*E*	37.7	n.i.	n.i.	n.i.	n.i.
26	EtO_2C		*E*	25.1	n.i.	n.i.	n.i.	n.i.
27	EtO_2C		*E*	n.i.	n.i.	n.i.	13.2	n.i.
28	EtO_2C		*E*	n.i.	n.i.	n.i.	n.i.	n.i.
29	EtO_2C		*E*	25.0	n.i.	n.i.	n.i.	32.0
30	EtO_2C		*E*	3.1	23.9	n.i.	20.8	n.i.
Hydroxy crotonic acid amide								
31	$HO\text{-}CH_2$	-CO-Gln-Leu-Val-Val-NH_2	*E*	27% (100 μM)	n.d. /	n.i.	n.d.	n.d.
Crotonic acid amide								
32	H_3C	-CO-Phe-OBn	*E*	20.3	n.i.	n.i.	n.i.	2.8
5-Hydroxy-pent-2-enoic acid esters								
33	EtO_2C		*E*	29.1	n.i.	36% (100 μM)	n.i.	19.0
Inhibitors with *Z*-configuration of the activated double bond 								
Maleic acid diesters								
34	EtO_2C		*Z*	54.3	n.i.	n.d.	100	17.0
Maleic acid amides								
35	EtO_2C	-CO-Gln(Trt)-OMe	*Z*	n.i.	n.d.	n.i.	30 % (10 μM)	25.5
36	EtO_2C	-CO-Phe-OBn	*Z*	26.1	n.i.	15% (100 μM)	28 % (100 μM)	8
37	HO_2C	-CO-Phe-OBn	*Z*	7.6	43.0	n.i.	100	n.i.
38	EtO_2C	-CO-Phe-Ala-OBn	*Z*	25.1	n.i.	43% (100 μM)	n.i.	2.7
39	HO_2C	-CO-Phe-Ala-OBn	*Z*	25.0	n.i.	n.d.	50.3	n.d.
40	EtO_2C	-CO-Phe-Ala-Leu-OBn	*Z*	6.6	36.8	29% (100 μM)	28.4	14.9
41	HO_2C	-CO-Phe-Ala-Leu-OBn	*Z*	10.5	n.i.	n.d.	14.6	n.d.

Table 1. Continued

Maleic acid amides								
42		-CO-Phe-OBn	*Z*	10.2	n.i.	0.15	100	2.9
43		-CO-Phe-Ala-OBn	*Z*	3.8	42.2	1.4	8.0	2.2
Vinylogous amino acid esters								
44	EtO_2C		*Z*	16.7	198	n.i.	100	n.i.
Amino isocrotonic acid esters								
45	EtO_2C		*Z*	n.i.	n.i.	47% (100 μM)	43.1	29.0
Reference compounds								
E-64				0.025	0.22	n.d.	3.2[a,c]	n.d.
Chloroquine				n.d.	n.d.	n.d.	0.07	n.d.
Melarsoprol				n.d.	n.d.	n.d.	n.d.	0.0026[b]
Eflornithine				n.d.	n.d.	n.d.	n.d.	30.7[b]
Suramine				n.d.	n.d.	n.d.	n.d.	0.31[b]
Nifurtimox				n.d.	n.d.	n.d.	n.d.	3.4[b]
Pentamidine				n.d.	n.d.	n.d.	n.d.	0.0029[b]

[a] Strain FCBR. [b] Taken from ref 29. [c] Strain FCBR: 5.3 μM.[28] [d] Chg, cyclohexyl glycine; Phg, phenylglycine; C1-NHAc, acetylaminoalanine; n.d., not determined; n.i., no inhibition at 100 μM; E-64, (S,S)-epoxysuccinyl-(S)-leucylamido(4-guanidino)butane. All values are mean values of at least two independent assays, with mean standard deviations less than 15% for antiprotease activity and less than 25% for antiparasitic activity unless otherwise explicitly indicated.

and **42**), studies to elucidate the inhibition mechanisms were performed.

Inhibitor **14** was subjected to dilution assays.[38] The residual activity of rhodesain was measured after 0, 5, 20, 35, and 50 min incubation times t_{inc} of enzyme and inhibitor prior to substrate addition ([S] = 10 μM). This was done for 15 different inhibitor concentrations of [I] = 0−100 μM. The inhibitor displayed time-independent inhibition. Additionally, these experiments were performed in the presence of 5 mM DTT. No differences in K_i values were found. Both experiments showed that inhibitor **14** is a reversible inhibitor that does not undergo nucleophilic Michael addition with either the enzyme or the low-molecular weight thiol DTT. The same experiments were performed with the *Z*-configured analogue **42**. These assays also showed inhibitor **42** to be a time-independent inhibitor.

In contrast, inhibitor **15** showed time-dependent inhibition of rhodesain in the dilution assays.[38] The second-order rate constant of inhibition k_{2nd} was calculated to be 11 100 M^{-1} s^{-1}. Again, inhibition was determined in the absence and presence of 5 mM DTT in the assay buffer. The K_i values,[39,40] which were determined using the slopes of the first minutes of progress curves obtained without preincubation of enzyme and inhibitor, were determined to 4.3 μM (without DTT, Table 1) and 3.6 μM (with DTT), showing that the compound does not react with low molecular weight thiols but only with the Cys residue of the enzyme. In order to exclude nonspecific inhibition by aggregation,[33] which could be due to the trityl moiety, the inhibition of falcipain-2 was also determined in the presence of various concentrations of the nonionic detergent Brij 35 (0.005%, 0.01%, 0.025%). No differences were found, excluding a nonspecific inhibition mechanism.

Table 2. Cytotoxicity Data for the Most Active Compounds[a]

compd	RD K_i (μM)	*T. b. b.* IC_{50} (μM)	J774.1 IC_{50} (μM)
12	7.6	0.25	3.5
15	4.3	2.8	> 100
16b	n.d.	3.5	60
19	n.i.	25.0	36.1
32	n.i.	2.8	45.2
33	36% (100 μM)	19.0	> 100
36	15% (100 μM)	8	3.3
38	43% (100 μM)	2.7	3.0
40	29% (100 μM)	14.9	29
42	0.15	2.9	31
43	1.4	2.2	32
45	47% (100 μM)	29.0	> 100

[a] RD, rhodesain; *T. b. b.*, *Trypanosoma brucei brucei*, J774.1, macrophage cell line; n.d., not determined; n.i., no inhibition at 100 μM.

Also, for the related inhibitors **16a** and **16b** time-dependent inhibition of rhodesain was observed. The k_{2nd} values were determined to be 12 250 M^{-1} s^{-1} (**16a**) and 26 483 M^{-1} s^{-1} (**16b**; k_i = 0.19 ± 0.019 min^{-1}; K_i = 0.14 ± 0.05 μM). A similar second-order rate constant k_{2nd} was found for inhibition of falcipain-2 by **16b**: 15 263 M^{-1} s^{-1} (k_i = 0.44 ± 0.053 min^{-1}; K_i = 0.49 ± 0.015 μM).

In order to prove that inhibition is competitive with respect to the substrate, K_i^{app} values for inhibition of rhodesain by **16a** were determined from the slopes of the first minutes of the progress curves obtained without preincubation of enzyme and inhibitor for various substrate concentrations ([S] = 5, 10, 20, 50, and 100 μM). The apparent dissociations constants K_i^{app} increased with increasing substrate concentrations, proving that inhibition is competitive.

A plot[41−43] of K_i^{app} vs [S] yielded a K_i value of 0.67 μM. In order to again exclude nonspecific inhibition by aggregation,[33] the K_i values for inhibition of rhodesain were determined in the absence and presence of nonionic detergent, both with the normally used enzyme concentration and with a 2-fold higher enzyme concentration, and additionally, the percentage of inhibition at 10 μM inhibitor concentration was determined after 0, 0.5, 2, and 5 min of spinning a solution containing enzyme and inhibitor in a microfuge. The experiments showed that the inhibition was independent of the absence or presence of nonionic detergent and independent of the enzyme concentration (K_i values: low enzyme concentration + detergent, 0.30 μM; low enzyme concentration without detergent, 0.64 μM; high enzyme concentration + detergent, 0.40 μM; high enzyme concentration without detergent, 0.64 μM) and also does not decrease after spinning the solution in a microfuge. Together with the observed competitive inhibition mechanisms, these results further prove that inhibition was not due to aggregation.

Inhibition of falcipain-2 by compound **20** was determined with either the substrate Cbz-Phe-Arg-AMC or Cbz-Leu-Arg-AMC. The K_i^{app} values were corrected to zero substrate concentration, yielding K_i values of 5.4 μM (Cbz-Phe-Arg-AMC) and 6.7 μM (Cbz-Leu-Arg-AMC), proving that inhibition is competitive with respect to the substrate.

Structure−Activity Relationships (SARs). The previous high-throughput on-bead screening[21] yielded fumaric acid peptides with Gln or Asn in P1-position and with Phg or Chg in P2-position or with Phe in P1-position as most potent inhibitors. Now we included compounds with these amino acids or respective peptides but modified acceptor-substituted alkene moieties. Thus, the following discussion of the SARs will consider the amino acids of the P1-position (Asn, Gln/Phe) and the Michael system.

Inhibitors with Asn or Gln. The fumaric acid diesters **6** and **7**, which contain the dipeptide moieties Asn-Phg and Gln-Phg instead of only Asn or Gln alone (compounds **4** and **5**), were more active against the enzymes and the parasites than **4** and **5**. Also, the fumaric acid diamides **8** and **10** containing only Asn or Gln were not active or only weakly active. Interestingly, inhibition potency against both enzymes and parasites was enhanced if the amino acid residues contained a trityl group, especially in the case of Gln (compounds **9** and **11**). This is also true for the vinylogous amino acid esters and dipeptides **15** and **16**, which were among the most active inhibitors against enzymes and parasites. Again, the additional Phg moiety in inhibitors **16** led to improvement. Notably, the minor diastereomer **16b** with (*R*)-Gln instead of (*S*)-Gln, which first could only be isolated in low yields but was resynthesized using (*R*)-Gln, displayed very high activity against the parasites and target proteases. Since a trityl group is a large lipophilic moiety that may lead to aggregation of the compounds, we examined the inhibition mechanisms of inhibitors **15** and **16a/b** (see above). All assays showed that inhibition was not due to aggregation but rather was time-dependent and competitive, indicating that the inactivation results from a Michael-type reaction between the Cys residue of the enzyme and the inhibitor. In a comparison of inhibitors **11** and **15**, the reversed peptide sequence (C−N in **15** vs N−C in **11**) and/or the shorter distance between the activated double bond and the Trt-proteced Gln residue seem to be superior for the inhibition of the proteases. Like inhibitors **4**, **5**, **8**, and **10**, also the amino crotonic acid esters **17** (Asn) and **18** (Gln), the hydroxy or aminoacrylic acid esters **21** (Gln), **22** (Asn), and **23** (Gln), and the hydroxycrotonic acid esters **24** (Asn) and **25** (Gln) were inactive or only weakly active. Prolongation of the peptide to Gln-Chg (**29**), Gln-Leu-Ala (**30**), or Gln-Chg-Val-Ala (**20**) again improved inhibition potency, at least against falcipains and plasmodia. This is in line with the good potency of the library derived heptapeptide inhibitors **2** and **3** (Figure 1, K_i values in M for falcipain-2: (**2**) 5.2; (**3**) 7.0), which are even more active. *Z*-Configuration of the activated double bond (compound **35** vs **11**) seems to be unfavorable. The inactivity of the Trt-containing *Z*-configured compound **35** compared to its *E*-analogue **11** additionally emphasizes that inhibition by **11** is not due to aggregation, since this should be independent from the configuration of the double bond.

Inhibitors with Phe. In the case of the Asn and Gln containing compounds, enzyme inhibition and antiparasitic activity correlated quite well; i.e., compounds not active against the enzymes did not inhibit parasite growth, and the most potent enzyme inhibitors (**11**, **15**, **16**, K_i values of 0.45−3.3 μM for FP-2, 1.4−14.7 μM for FP-3, and 0.14−4.3 μM for RD) were also quite active against both plasmodia and trypanosomes (IC_{50} of 0.09−4.7 for plasmodia, 2.8−31 μM for trypanosomes). In contrast, some of the Phe containing compounds displayed strong enzyme inhibition while being inactive against parasites or were active against parasites while being inactive or only weakly active against the target enzymes.

Results showing inhibition of parasite growth without inhibition of proteases especially concern rhodesain/trypanosoma; examples are compounds **19**, **32**, **33**, **36**, **38**, **40**, and **45**. These results raise a question regarding the target of the antitrypanosomal activity of these compounds.

On the other hand, highly active enzyme inhibitors did not display antiparasitic activity. The striking example for rhodesain/trypanosomes is compound **14**, which was equally potent to its *Z*-isomer **42** (K_i = 0.34 μM/0.15 μM) but in contrast to the latter did not display antitrypanosomal activity up to 100 μM. Compound **14** also exhibits good inhibition of falcipains (K_i = 0.98, 2.8 μM) but not against plasmodia. As described above, **14** and **42** are reversible inhibitors. Interestingly, replacement of the ester moiety in inhibitor **12** by Phe benzyl ester (**14**) totally abolishes antitrypanosomal activity but on the other hand improves enzyme inhibition. In the case of the corresponding *Z*-isomers (**36** vs **42**), however, both, enzyme inhibition and parasite inhibition were improved. Polar groups like a free acid (inhibitors **13**, **37**, **41**) or the hydroxymethyl group in **31** decreased antiprotease and antiparasitic activity.

Among the various Michael systems (compare *E*-configured inhibitors **12**, **19**, **32**, **33** or *Z*-configured inhibitors **34**, **36**, **44**, **45**), the fumaric acid ester **12** and the corresponding maleic acid ester **36** were best concerning antitrypanosomal activity.

In summary, the results show that there are some quite good starting points for falcipain (**12**, **14**, **43**) and rhodesain (**12**, **14**, **42**, **43**) inhibitors on the one hand and for antitrypanosomal (**12**, **32**, **38**, **42**, **43**) and antiplasmodial (**43**) activity on the other hand, with inhibitor **43** displaying the best overall activity within the Phe containing compound series.

Toxicity Studies. The most active antitrypanosomal compounds, namely, **12**, **15**, **16b**, **42**, **43** as well as those that were active against trypanosomes but not against rhodesain (**19**, **32**, **33**, **36**, **38**, **40**, **45**), were subjected to cytotoxicity assays

with the macrophage cell line J774.1. Results are shown in Table 2, which additionally contains the data for inhibition of rhodesain and trypanosomes.

These studies show that in the case of the Phe containing compounds displaying antitrypanosomal activity but lacking good rhodesain inhibition, nonspecific cytotoxicity may indeed be one reason for the antiparasitic activity (compounds **19**, **32**, **36**, **38**, **40**). Selectivity indices (IC_{50}(J774.1)/IC_{50}(*T.b.b.*)) of about 10 are found for inhibitors **12**, **42**, and **43**. In contrast, the Gln(Trt) containing inhibitors are only weakly toxic (**16b**: 60 μM) or do not show nonspecific toxicity below 100 μM (**15**), yielding selectivity indices of 17 and >36.

Summary and Conclusion

Starting from the results of a combinatorial fumaric acid based peptide library that yielded peptides with Phe, Asn, or Gln in P1-position to display high inhibitory potency against falcipain-2 and rhodesain, we synthesized and tested a series of analoguous peptides containing various Michael acceptors. Both, *E*- and *Z*-configured compounds were included and tested against the target enzymes (falcipain-2, falcipain-3, rhodesain), the parasites (*Plasmodium falciparum*, *Trypanosoma brucei brucei*), and macrophages. In the case of *E*-configured compounds, those with a Gln(Trt) residue, namely, **11**, **15**, **16a**, and **16b**, displayed the highest protease inhibiting and antiparasitic properties with the diastereomeric vinylogous dipeptide esters **16a** and **16b** (dipeptide sequence (*S*/*R*)-Gln(Trt)-Phg) as the most active inhibitors in the whole series (**16a**(**b**) K_i (FP-2/FP-3/RD) = 0.45(0.49)/1.4(nd.)/0.47(0.14) μM; **16a**(**b**) IC_{50} (*P. falciparum*/*T. brucei*) = 3.9 (0.9)/31 (3.5) μM). Inhibition of the target enzymes was shown to be time-dependent (**15**/**16a**/**16b**: k_{2nd} = 11100/12250/15263 (FP-2), **16b**: 26483 (RD) M^{-1} s^{-1}) and competitive with respect to the substrate. Unspecific inhibition by aggregation was excluded by various experiments (addition of detergent, increased enzyme concentration, spinning in a microfuge). The analoguous *Z*-configured compound was inactive. The most active inhibitors, especially against rhodesain and *Trypanosoma*, among the *Z*-configured compounds are the Phe-containing maleic acid derivatives **42** and **43** (K_i (FP-2/RD) = 10.2/3.8 μM for **42** and 3.8/1.4 μM for **43**; IC_{50} (*P. falciparum*/*T. brucei*) = 100/2.9 μM for **42** and 8.0/2.2 μM for **43**). However, in contrast to the Gln derivatives described above, which were shown to be not toxic, unspecific cytotoxicity may also be a reason for the antitrypanosomal activity (IC_{50} (macrophages) $\approx$ 30 μM). Interestingly, the *E*-configured analogue **14** displayed even a bit higher protease inhibition (K_i (FP-2/FP-3/RD) = 0.98/2.8/0.34) but no antiparasitic activity. Both the *E*-configured (**14**) and the *Z*-configured derivatives (**42**) showed time-independent, reversible, and competitive inhibition. In summary, we have discovered new peptidic Michael-type cysteine protease inhibitors with promising properties displaying different inhibition mechanisms depending on the configuration of the double bond and the peptide sequence: on the one hand, the *E*-configured vinylogous Gln(Trt) esters as time-dependent inhibitors, and on the other hand, *Z*- or *E*-configured maleic and fumaric acid derivaites containing two Phe residues as reversible inhibitors. The Gln(Trt) derivatives especially are promising starting points for new inhibitors, since they display high antiparasitic activity while being nontoxic against macrophages (selectivity index of >36).

Experimental Section

Biological Activities. General. Falcipains and rhodesain were recombinantly expressed as described previously (FP-2 in ref 31, FP-3 in ref 44, and RD in ref 45). Substrates (Cbz-Phe-Arg-AMC for rhodesain, Cbz-Leu-Arg-AMC for falcipains) were purchased from Bachem. Assay buffer was 50 mM acetate, pH 5.5, 5 mM EDTA, 200 mM NaCl. Enzyme buffer was 50 mM acetate, pH 5.5, 5 mM EDTA, 200 mM NaCl, 5 mM DTT. Substrates and inhibitor stock solutions were prepared in DMSO and diluted with assay buffer (final DMSO concentration of 10%). A Varian Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) with 96-well plates was used: λ_{ex} = 380 nm and λ_{em} = 460 nm.

Determination of K_i Values. The hydrolyses of the substrates were monitored over 5−10 min in the presence of inhibitor. The K_i values were calculated using the Dixon equation,[41] $v_o/v_i = 1 + ([I]/K_i^{app})$, and correction to zero substrate concentration[41] was done with $K_i = K_i^{app}/(1 + [S]/K_m])$ with [S] = 25 μM and K_m = 8.4 μM for falcipain-2, with K_m = 72 μM for falcipain-3, and with [S] = 10 μM and K_m = 827 nM for rhodesain. GraFit software was used to calculate the K_i^{app} values.

Determination of k_{2nd} Values. The residual activities of rhodesain were measured after 0, 5, 20, 35, and 50 min of incubation time t_{inc} of inhibitor (**15**, **16a**) and enzyme prior to addition of substrate ([S] = 10 μM). The residual enzyme activities v_i for various inhibitor concentrations ranging between 0 and 100 μM were fitted to the incubation times t_{inc} using equation $v_i = v_o \exp(-k_{obs}t_{inc}) + \text{constant}$. The thus obtained pseudo-first-order rate constants k_{obs} were plotted against the inhibitor concentrations. The graphs were observed to be linear; thus, the second-order rate constants of inhibition k_{2nd} were calculated by the slopes of the curves, with correction to zero substrate concentration: $k_{2nd} = (1 + [S]/K_m)(k_{obs}/[I])$. In the case of inhibitor **16b**, continuous assays with rhodesain and falcipain-2 were performed, and k_{obs} values were obtained by fitting the progress curves to the exponential equation $y = \lim(1 - \exp(-k_{obs}t)) + \text{offset}$. The thus obtained pseudo-first-order rate constants k_{obs} were plotted against the inhibitor concentrations. The graphs showed a hyperbolic shape, and the second-order rate constants of inhibition were thus calculated using equations $k_{obs} = k_i[I]/(K_i^{app} + [I])$ (yielding the first-order rate constant k_i and the apparent dissociation constant K_i^{app}) and $k_{2nd} = (1 + [S]/K_m)(k_i/K_i^{app})$. GraFit software was used to calculate the inhibition constants.

Evaluations of cultured malaria parasites were as described previously.[12,28,35]

Antitrypanosomal activities and studies on macrophages were as described previously.[29]

Syntheses of Inhibitors. General. Reaction solvents were dried and distilled before use. All other reagents were used as obtained from Aldrich, Fluka, IRIS, or GL Biochem. All experiments were run in oven-dried glassware under N_2 atmosphere. NMR spectra were recorded on an Avance 400 MHz spectrometer from Bruker Biospin GmbH, Germany (solvent $CDCl_3$; ^{1}H NMR, 400.13 MHz; ^{13}C NMR, 100.61 MHz). Peak assignments are based on DEPT. ESI mass spectra were recorded on an Agilent 1100 LC/MSD trap. Characterization of the purity of the compounds by LC analyses was done with the following parameters: HPLC system 1100 from Agilent and a Jupiter 4 μm Proteo 90A RP C-18 column (4.6 mm × 150 mm), gradient from 40% acetonitrile with 0.1% formic acid (5 min), 40−95% acetonitrile with 0.1% formic acid (25 min) and 95% acetonitrile with 0.1% formic acid for 15 min, flow rate of 600 μL/min, and UV detection at 220 and 254 nm. The purities of the compounds are generally $>98\%$ unless otherwise indicated. Melting points (not corrected) were determined in open capillary on a type 510 apparatus from Büchi, Switzerland. IR spectra were recorded on a FT-IR spectrometer, type PharmalyzIR, from Bio-Rad. The α values were detemined on a 241

polarimeter from PerkinElmer. Column chromatography (cc) was performed with silica gel 60 from Merck (0.063−0.2 mm or 70−230 mesh). For TLC (thin layer chromatography) alumina sheets from Merck coated with silica gel 60 F_{254} were used. All amino acids used are L-configured.

Synthetic Methods. Method A (Steglich Esterification). An amount of 1.00 equiv of carboxylic acid was suspended in $CH_2Cl_{2\,abs}$ (~5 mL/mmol) and cooled to 0 °C. Then 1.10 equiv of alcohol, 0.08−0.10 equiv of DMAP, and 1.10 equiv of DCC were added. The reaction mixture was stirred at rt for 24 h, and the precipitated dicyclohexylurea was filtered off. The organic phase was washed with saturated solutions of $NaHCO_3$, NH_4Cl, and NaCl and dried with sodium sulfate, the solvent was removed in vacuo, and the residue was purified by cc.

Method B (Boc Deprotection and Peptide Coupling with DPPA). An amount of 1.00 mmol of Boc-protected compound was dissolved in 6 mL of dichloromethane and cooled to 0 °C. An amount of 6 mL of TFA was added, and the mixture was stirred at 0 °C. After completion of the deprotection (TLC control) the solvent was removed in vacuo, and the remaining residue was dried and directly submitted to peptide coupling. Amounts of 1.00 equiv of acid and 1.05 equiv of amine dissolved in DMF were cooled to 0 °C. An amount of 1.10 equiv of DPPA was added, and the reaction mixture was stirred for 30 min. Then an amount of 2.05 equiv of TEA was added, and the mixture was stirred for 18−36 h at 0 °C and for 2−5 days at rt (TLC control). Water was added, and the reaction mixture was extracted with ethyl acetate.The organic phase was washed with saturated NH_4Cl, $NaHCO_3$ and NaCl solutions, dried, and removed in vacuo. The crude product was purified by cc.

Method C (Symmetric Anhydride Method). An amount of 2.20 equiv of acid was dissolved in dichloromethane and cooled to 0 °C. Then an amount of 1.10 equiv of DCC was added and the mixture was stirred for 1 h. Precipitated dicyclohexylurea was filtered off, and 1.00 equiv of C-protected amine was added to the filtrate (in the case of hydrochlorides or tosylates 1.00 equiv of TEA was added additionally) followed by 0.10 equiv of DMAP. The reaction mixture was stirred at 0 °C for 6−10 h and at rt for 1 day (TLC control). The reaction mixture was washed with saturated $NaHCO_3$, $KHSO_4$ solutions, and water, dried, and removed in vacuo. The crude product was purified by cc.

Method D (Coupling with IBCF). An amount of 1 equiv of acid was dissolved at −15 °C in THF, and 1 equiv of NMM and 1 equiv of IBCF were added successively. Then 1 equiv of amino acid dissolved in DMF was added, and the reaction mixture was stirred at −15 °C for 1 h, at 0 °C for 1 − 2 h, and at rt for 10−18 h (TLC control). The mixture was filtered off, and the solvent was removed in vacuo. The remaining residue was dissolved in ethyl actetate. The organic phase was washed with saturated NH_4Cl, $NaHCO_3$, and NaCl solutions, dried, and removed in vacuo. The crude product was purified by cc.

Method E (Coupling with PyBOP). Amounts of 1 equiv of acid, 3 equiv of NMM or DIPEA, and 1 equiv of PyBOP were dissolved in DMF and stirred at rt for 15−30 min. The amine (1 equiv) was added, and the solution was stirred for 1−3 days (TLC control). The reaction mixture was poured into ethyl acetate and washed with saturated NaCl solution. The organic phase was dried, filtered off, and removed in vacuo. The crude product was purified by cc.

Method F (Michael Addition of Alcohols or Amines to Ethyl Propiolate). An amount of 1.00 mmol (1.00 equiv) of alcohol or amino acid was dissolved in $CHCl_3$ and cooled to 0 °C. Catalytic amounts of TEA (0.05−0.1 mL) and 1.20 mmol (1.20 equiv) of ethyl propiolate were added successively. The solution was stirred for 1 day at rt and quenched by addition of saturated NH_4Cl solution. The mixture was poured into 70 mL of $CHCl_3$, and the organic phase was washed with saturated NH_4Cl and NaCl solutions, dried, and evaporated. The crude product was purified by cc. Hydrochlorides or tosylates of amino acids were first reacted with $NaHCO_3$ and extracted with ethyl acetate to yield the free amines.

Method G (Reaction of Maleic Anhydride with Amino Acids or Peptides). To a mixture of amino acid, di- or tripeptide (1 equiv), and NEt_3 (2.1 equiv) in methylene chloride, maleic anhydride (1 equiv) was added at 0 °C. The solution was stirred at 0 °C for 2 h and at rt for a further 24 h. The solution was acidified to pH 2 with 2 M HCl and extracted with methylene chloride. The combined organic phases were washed with saturated NaCl solution and dried over Na_2SO_4, and the solvent was evaporated to yield the *Z*-olefin. The crude product was purified by cc.

Syntheses and Analytical Data of the Selected Inhibitors 11, 14, 15, 16a, 16b, 42, 43. (*S*,*E*)-Methyl 2-(4-Ethoxy-4-oxobut-2-enamido)-5-oxo-5-(tritylamino)pentanoate (11). Method B with 75.0 mg (522 μmol) of monoethyl fumarate, 200 mg (497 μmol) of H-Gln(Trt)-OMe, 118 μL (151 mg, 547 μmol) of DPPA, and 73 μL (53 mg, 522 μmol) of TEA in 5 mL of DMF_{abs} was used. Purification was done by cc (silica gel 60, cyclohexane/ethyl acetate 1/1. Yield: 56 mg (106 μmol, 21%), colorless solid. Mp: 138−141 °C. $[\alpha]_D^{21}$ −13.46 (*c* 0.56, MeOH). ESI-MS (*m*/*z*): calcd for $C_{31}H_{32}N_2O_6$, 528.23 [M]; found, 529.0 [M + 1], 551.5 [M + Na^+]. ^{1}H NMR ($CDCl_3$): δ (ppm) = 7.16−7.35 (m, 16 H), 6.88 (s, 1 H), 6.73 (d, 1 H, *J* = 15.7 Hz), 6.68 (d, 1 H, *J* = 15.7 Hz), 4.49−4.54 (m, 1 H), 4.25 (q, 2 H, *J* = 7.3 Hz), 3.70 (s, 3 H), 2.36−2.49 (m, 2 H), 2.14−2.20 (m, 1 H), 1.99−2.06 (m, 1 H), 1.30 (t, 3 H, *J* = 7.3 Hz). IR (neat): $\tilde{\nu}$ = 3302, 2962, 1723, 1659, 1516, 1492, 1446, 1367, 1297, 1260, 1171, 1094, 1032 cm^{-1}.

(*S*)-2-[(*E*)-3-((*S*)-1-Benzyloxycarbonyl-2-phenylethylcarbamoyl)-acryloylamino]-3-phenylpropionic Acid Benzyl Ester (14). Amounts of 584 mg (2.00 mmol) of PheOBn·HCl and 0.29 mL (2.10 mmol) of TEA were dissolved in DMF, and 0.11 mL (1.00 mmol) of fumaric acid dichloride was added dropwise at −15 °C. The mixture was stirred at −15 °C for 1 h, at 0 °C for 1 h, and at rt for an additional 1 h. The solution was poured into an ethyl acetate/water mixture (1/1). The aqueous phase was extracted with ethyl actetate, and the combined organic phases were washed with brine, dried, and evaporated in vacuo. The crude product was recrystallized from dichoromethane/cyclohexane. Yield: 61 mg (0.27 mmol, 27%), colorless solid. Mp: 215 °C. $[\alpha]_D^{22}$ +5.40° (*c* 0.50, $CHCl_3$). LOOP-ESI-MS: calcd for $C_{36}H_{34}N_2O_6$, 590.68; found, [M + Na]$^+$ 613.7. ^{1}H NMR ($CDCl_3$): δ = 3.10−3.21 (m, 4 H, *J* = 5.8, 8.4 Hz), 4.88−4.93 (dt, 2 H, *J* = 5.8, 7.9 Hz), 5.14, 5.16 (2d, 4 H, *J* = 12.1 Hz), 6.29 (d, 2 H, *J* = 7.9 Hz), 6.83 (s, 2 H), 7.18−7.39 (m, 20 H) ppm. IR (neat): $\tilde{\nu}$ = 3296, 3031, 2941, 1728, 1677, 1627, 1535, 1443, 1352 cm^{-1}.

(*S*,*E*)-Ethyl 4-(*tert*-Butoxycarbonylamino)-7-oxo-7-(tritylamino)-hept-2-enoate (15).[46] Reduction with DIBAL-H was done with 300 mg (600 μmol) of Boc-Gln(Trt)-OMe and 6.0 mL (6.00 mmol) of DIBAL-H (1.0 M in hexane) in 10 mL of $CH_2Cl_{2\,abs}$ for 3 h. The crude aldehyde was subjected to olefination: 268 mg (567 μmol) of aldehyde, 27.5 mg (624 μmol) of anhydrous LiCl, 135 μL (153 mg, 680 μmol) of triethylphosphonoacetate, and 96.4 μL (73.3 mg, 567 μmol) of DIPEA in 6−8 mL of CH_3CN_{abs} were mixed at 0 °C and stirred at rt for 1 day. An amount of 10 mL of NaCl solution was added. The reaction mixture was acidified to pH 6 with 2 M HCl and extracted with ethyl acetate. The combinded organic phases were washed with saturated $NaHCO_3$, NH_4Cl, and NaCl solutions, dried, and evaporated in vacuo. The crude product was purified by cc (silica gel 60, gradient cyclohexane/ethyl actetate 4/1 → 3/1). Yield: 130 mg (240 μmol, 42%), colorless solid. Analytical data corresponded to literature values.[46] Mp: 147−150 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) = 7.18−7.30 (m, 15 H), 6.87 (br s, 1 H, NH), 6.79 (dd, 1 H, *J* = 5.3 Hz, *J* = 15.7 Hz), 5.88 (dd, 1 H, *J* = 1.5 Hz, *J* = 15.7 Hz), 4.76 (br d, 1 H, *J* = 6.6 Hz), 4.29 (br s, 1 H), 4.18 (q, 2 H, *J* = 7.1 Hz), 2.36 (m_c, 2 H), 1.89−1.99 (m, 1 H), 1.71−1.80 (m, 1 H), 1.42 (s, 9 H), 1.27 (t, 3 H, *J* = 7.1 Hz). IR (neat): $\tilde{\nu}$ = 3306, 2926, 1687, 1656, 1518, 1492, 1447, 1366, 1249 cm^{-1}.

(*S*,*E*)-Ethyl 4-((*S*/*R*)-2-(*tert*-Butoxycarbonylamino)-2-phenyl-acetamido)-7-oxo-7-(tritylamino)hept-2-enoate (16a, 16b). Reduction with DIBAL-H was done with 500 mg (787 μmol) of Boc-Phg-Gln(Trt)-OMe in 20 mL of $CH_2Cl_{2\,abs}$ and 7.9 mL (7.87 mmol) of

DIBAL-H (1.0 M in hexane) for 7 h. The crude aldehyde was subjected to olefination. An amount of 41.0 mg (944 μmol) of anhydrous LiCl was suspended in 15 mL of CH_3CN_{abs} at rt. Successively 187 μL (212 mg, 944 μmol) of triethylphosphonoacetate, 118 μL (120 mg, 787 μmol) of DBU, and a solution of 497 mg (787 μmol) of Boc-Phg-Gln(Trt)-H in 20 mL of CH_3CN_{abs} were added. The reaction mixture was worked up as described for **15**. Purification was done by cc (silica gel 60, gradient *n*-hexane/EtOAc = 2:1 → 1.5:1). Yield: **16a**, 57 mg (84.3 μmol, 11%); **16a** + **16b**, 122 mg (181 μmol, 23%); **16b**, 20 mg (29.6 μmol, 4%). The Horner−Wadsworth−Emmons olefination also yielded the diastereomeric mixture (**16b**/**16a** = 20/80). Compound **16b** was resynthesized using Boc-Phg-(*R*)-Gln(Trt)-OMe. The purification was performed by preparative HPLC. Yield: 110 mg, 10%. Mp: (**16a** + **16b**) 159−162 °C. $[\alpha]_D^{21}$ +6.65 (**16a**, *c* 0.29, MeOH). ESI-MS (**16a**) (*m*/*z*): calcd for $C_{41}H_{45}N_3O_6$, 675.33 [M]; found, 676.4 [M + 1], 698.3 [M + Na^+]. ^{1}H NMR ($CDCl_3$/D_2O, **16a**): δ (ppm) = 7.15−7.31 (m, 20 H), 6.64 (dd, 1 H, *J* = 4.6 Hz, *J* = 15.7 Hz), 5.28−5.35 (m, 1 H), 4.97 (br s, 1 H), 4.49−4.52 (m, 1 H), 4.09 (q, 2 H, *J* = 7.1 Hz), 2.32−2.39 (m, 2 H), 1.58−1.95 (m, 2 H), 1.38 (br s, 9 H), 1.22 (t, 3 H, *J* = 7.1 Hz). ^{1}H NMR ($CDCl_3$/D_2O, **16b**): δ (ppm) = 7.15−7.31 (m, 20 H, *J* = Hz), 6.75 (dd, 1 H, *J* = 4.9 Hz, *J* = 12.5 Hz), 5.73 (dd, 1 H, *J* = 12.5 Hz, *J* = 10.0 Hz), 4.93 (m, 1 H), 4.49−4.51 (m, 1 H), 4.11 (q, 2 H, *J* = 7.0 Hz), 2.56−2.65 (m, 1 H), 2.38−2.56 (m, 2 H), 2.29−2.18 (m, 1 H), 1.39 (br s, 9 H), 1.21 (t, 3 H, *J* = 7.0 Hz). $[\alpha]_D^{20}$ +2.20 (**16b**, *c* 0.99, MeOH). ESI-MS (**16b**) (*m*/*z*): calcd for $C_{41}H_{45}N_3O_6$, 675.33 [M]; found, 676.5 [M + 1], 698.5 [M + Na^+]. IR (neat, **16a** + **16b**): $\tilde{\nu}$ = 3279, 2917, 2850, 1716, 1644, 1519, 1492, 1410, 1366, 1272, 1165, 1035 cm^{-1}.

(*S*)-Benzyl-2-[(*Z*)-3-((*S*)-1-benzyloxycarbonyl-2-phenylethylcarbamoyl)acryloylamino]-3-phenylpropionate (42). Method E with 354 mg (1.00 mmol) of **37**, 292 mg (1.00 mmol) of Phe-OBn·HCl, 521 mg (1.00 mmol) of PyBOP in 10 mL of dichlormethane/DMF (1/1), and 0.52 mL (3.00 mmol) of DIPEA was used. Reaction time: 3 h, 0 °C, 5 days, rt. Purification was done by cc (silica gel 60, cyclohexane/EtOAc 2/1). Yield: 175 mg (0.30 mmol, 30%) yellowish resinous solid. $[\alpha]_D^{22}$ +57.53° (*c* 0.73, $CHCl_3$). LOOP-ESI-MS: calcd for $C_{36}H_{34}N_2O_6$, 590.68; found, [M + Na]$^+$ 613.7. ^{1}H NMR ($CDCl_3$): δ = 3.10−3.21 (m, 4 H, *J* = 6.3 Hz), 4.88−4.93 (dt, 2 H, *J* = 6.3, 7.0 Hz), 5.15, 5.16 (2d, 4 H, *J* = 12.1 Hz), 6.06 (s, 2 H), 7.08−7.34 (m, 20 H), 8.51 (d, 2 H, *J* = 7.0 Hz) ppm. IR (neat): $\tilde{\nu}$ = 3250, 3031, 2955, 1740, 1671, 1616, 1537, 1454, 1347, 1260 cm^{-1}.

(*S*)-Benzyl-2-{(*Z*)-3-[(*S*)-1-((*S*)-1-benzyloxycarbonylethylcarbamoyl)-2-phenylethylcarbamoyl]acryloylamino}-3-phenylpropionate (43). Method E with 211 mg (0.55 mmol) of **37**, 242 mg (0.55 mmol) of Phe-Ala-OBn·TFA and 286 mg (0.55 mmol) of PyBOP in 20 mL of dichlormethane/DMF (1/1), and 0.28 mL (1.65 mmol) of DIPEA was used. Reaction time: 5 h, 0 °C, 5 days, rt. Purification was done by cc (silica gel 60, cyclohexane/ethyl acetate 1/1). Yield: 147 mg (0.22 mmol, 40%) yellowish resinous solid. $[\alpha]_D^{22}$ +1.28° (*c* 0.47, $CHCl_3$). LOOP-ESI-MS: calcd for $C_{39}H_{39}N_3O_7$, 661.76; found, [M + Na]$^+$ 684.7. ^{1}H NMR ($CDCl_3$): δ = 1.35 (d, 3 H, *J* = 7.3 Hz), 3.08−3.18 (m, 4 H, *J* = 6.0, 6.8 Hz), 4.51−4.58 (dt, 1 H, *J* = 7.1, 7.3 Hz), 4.67−4.74 (dt, 1 H, *J* = 6.8, 7.8 Hz), 4.84−4.91 (dt, 1 H, *J* = 6.0, 7.3 Hz), 5.13, 5.15 (2d, 4 H, *J* = 12.1 Hz), 6.03 (d, 1 H, *J* = 13.2 Hz), 6.08 (d, 1 H, *J* = 13.2 Hz), 6.81 (d, 1 H, *J* = 7.3 Hz), 7.16−7.40 (m, 20 H), 7.94 (d, 1 H, *J* = 7.3 Hz), 8.41 (d, 1 H, *J* = 7.8 Hz) ppm. IR (neat): $\tilde{\nu}$ = 3273, 3063, 3032, 2929, 1741, 1658, 1621, 1536, 1497, 1452, 1385, 1260 cm^{-1}.

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Supporting Information Available: Synthetic methods and analytical data (NMR, IR, MS, purity, optical rotation, melting points) for the synthesized compounds and ^{13}C NMR data for compounds **11**, **14**, **16a**, **16b**, **42**, **43**. This material is available free of charge via the Internet at http://pubs.acs.org.

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