

Peptide derivatives specific for a Plasmodium falciparum proteinase inhibit the human erythrocyte invasion by merozoites

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Addition of a drug solution or saline/10% EG (25 μL) was followed with incubation over 60 min at 37 °C. Then the aggregating agent (25 μL; 2 μM ADP, 0.1 μM adrenaline, 2 μg/mL collagen, 1.4 μM AA) was added and the aggregating effect carried out over 5 min with stirring at a controlled temperature of 37 °C. Appropriate concentrations of aggregating agents were determined by an initial titration. Each compound was studied with several normal plasmas.

The extent of aggregation of test samples was compared with the extent of aggregation of control samples and is expressed as "percent of control".

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Peptide Derivatives Specific for a *Plasmodium falciparum* Proteinase Inhibit the Human Erythrocyte Invasion by Merozoites

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A specific proteinase of *P. falciparum* merozoites has been detected by using hydrosoluble fluorogenic peptidic substrates synthesized by classical peptide chemistry; their N-terminal end was acylated by a gluconoyl group that protects them from aminopeptidase degradation and increases their hydrosolubility, and their carboxylic end was substituted by a 3-amino-9-ethylcarbazole group. The sequence Val-Leu-Gly-Lys was found to be the most specific substrate. On this basis, reversible peptidic inhibitors were synthesized by substituting the C-terminal lysyl residue, at the proteolytic site, by different alkylamines and amino alcohols. The activity of these compounds, studied on the *P. falciparum* proteinase and in in vitro cultures, strongly suggests a specific effect of this peptidic sequence on the reinvasion process. The peptidic inhibitors do not impair the release of merozoites from schizonts, but selectively inhibit the invasion step leading to the formation of rings. Although the natural target of this enzyme is not yet known, these specific peptide inhibitors could lead to a new antimalarial approach.

Introduction

The dramatic worldwide resurgence of malaria recently observed is mainly due to the spreading of antimalarial drug-resistant parasite strains and it is now clear that new therapeutic approaches are required. Researches to develop antimalarial vaccines are steaming ahead but several years will probably pass before this strategy will be an effective protection of human beings.

As the invasion of red blood cells by merozoites is a key event during malarial infection, the inhibition of this step appears as an attractive biological approach. In order to understand the molecular aspect of the highly efficient invasiveness of merozoites into erythrocytes, we were looking for specific parasite proteinases using various fluorogenic peptidic substrates. Proteinases have indeed been implicated in different steps of the *Plasmodia* life cycle and particularly in the release of merozoites and the invasion of erythrocytes (see Schrevel et al.[1], for a review).

A neutral 68 kDa proteinase has been isolated in *Plasmodium berghei* (Pb 68)[2] and in *P. falciparum* (Pf 68)[3] schizonts. Its localization in the apex of *P. berghei* merozoites,[4] as well as its presence in free *P. falciparum* merozoites, arised the question of the biological role of this proteinase. As parasite proteinases could act on host erythrocyte proteins, the role of the Pf 68 proteinase during the reinvasion process of erythrocytes by merozoites was investigated.

. In the present paper, the synthesis of substrates specific for this *P. falciparum* proteinase is described: their N-terminal end was acylated by a hydrosolubilizing gluconoyl group[5] and their C-terminal end, at the proteolytic cleavage site, was substituted by a 3-amino-9-ethylcarbazole group (AEC). This fluorescent amine has been selected among several fluorescent reporter groups such as 4-methoxy-2-naphthylamine,[6] 7-amino-4-methylcoumarin,[7] or 7-amino-4-(trifluoromethyl)coumarin[8] for its interesting and particular spectroscopic properties,[9] its high sensitivity, and its very good coupling yield with amino acids. Furthermore, this amine allows quantitative determination of proteinases in cell lysates and/or supernatants.[10]

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Table I. Kinetic Constants of the Proteinase of *P. falciparum* for the Different Fluorogenic Substrates Synthesized

no.	substrates P_5	P_4	P_3	P_2	P_1	P_1'	K_m, mM	V_{rel}[a]
S-1	GlcA	Val	Leu	Gly	Lys	AEC	0.38	100
S-2	GlcA	Val	Leu	Gly	Arg	AEC	0.40	89
S-3		GlcA	Ser	Gly	Lys	AEC	0.73	140
S-4	GlcA	Thr	Leu	Gly	Lys	AEC	0.77	74
S-5	GlcA	Ile	Val	Gly	Lys	AEC	0.95	43
S-6	GlcA	Ser	Leu	Gly	Lys	AEC	1.14	17
S-1a	Boc	Val	Leu	Gly	Lys	AEC	1.14	11
S-7	GlcA	Val	Thr	Gly	Lys	AEC	1.25	26
S-8	GlcA	Val	Leu	Sar	Lys	AEC	1.67	80
S-9	GlcA	Ala	Ala	Ala	Lys	AEC	5.00	29
S-10		GlcA	Thr	Gly	Lys	AEC	5.60	23
S-11	GlcA	Ile	Val	Gly	Arg	AEC	6.25	3
S-1b	Z	Val	Leu	Gly	Lys	AEC	>10	<3
S-1c	H	D-Val	Leu	Gly	Lys	AEC	>10	<3
S-12		GlcA	Ser	Gly	Arg	AEC	>10	<3
S-13		GlcA	Ser(Bzl)	Gly	Lys	AEC	>10	<3
S-14		GlcA	Val	Phe	Lys	AEC	>10	<3

[a] V_{rel} is the relative rate of hydrolysis of the substrates determined in Tris-HCl (50 mmol), pH 7.4, at 37 °C. For S-1 the reaction velocity V_m was 35 μmol/mL per s.

The specificity of the substrates was determined by monitoring the release of the fluorescent amine in the presence of the *P. falciparum* purified proteinase. From these results, specific peptidic inhibitors were synthesized. The rationale of this method consists of the design of compounds which, due to their affinity for the active site, can compete preferentially with the natural substrate for reversible active-site occupancy, thus impeding the proteolytic process. The activity of these compounds was monitored by measuring their capacity to inhibit the substrate hydrolysis by the *P. falciparum* proteinase and the invasion of red blood cells in in vitro experiments.

Results

Chemical Synthesis. By using a large panel of peptidyl-3-amido-9-ethylcarbazole substrates, a highly selective proteinase for the Val-Leu-Gly-Arg (VLGR) sequence has been demonstrated in the endoerythrocytic stages of *P. berghei* and *Plasmodium chabaudi*.[2] In order to check whether this enzyme of rodent malarial parasites is also present in *P. falciparum* and whether the active site of these proteinases is conserved among different *Plasmodia* species, several analogues of the Val-Leu-Gly-Arg sequence were synthesized.

Conventionally, the amino acid sequence of the proteinase cleavage site is denoted as $H_2N...P_4\text{-}P_3\text{-}P_2\text{-}P_1\text{-}P_1'\text{-}P_2'...COOH$, where the cleavable bond is between amino acids P_1 and P_1'.[11] Taking into account that the merozoite proteinase hydrolyzes specifically the substrates after a basic P_1 residue and that the glycine residue in P_2-position is essential,[2] the substrates were synthesized by a racemization-free fragment coupling strategy, involving the carboxylic group of the C-terminal glycine of di- or tripeptides and Lys(Fmoc)-AEC or Arg-AEC.

N^{α}-Boc or N^{α}-Z peptidic substrates bearing a fluorophore group such as aminomethylcoumarin or aminoethylcarbazole are generally only scarcely soluble in water. The hydrosolubility of such fluorogenic substrates can be improved when their N-terminal amino group is either free or succinylated due to the presence of a positive or negative charge, respectively. In order to avoid such charges and their putative effects in lowering the binding affinity, the N-terminal end of the substrates was acylated by a neutral polyhydroxyalcanoyl group,[5] which also protects the peptides from aminopeptidase degradation. The gluconoyl group has been selected for its good hydrosolubilizing capacity and for its low cost.

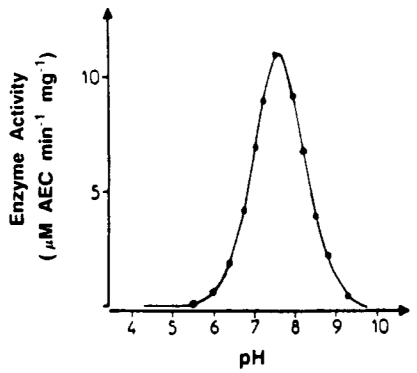


Figure 1. Effect of the pH on the hydrolysis of the substrate GlcA-Val-Leu-Gly-Lys-AEC by the *P. falciparum* proteinase. The enzyme activity was determined in 100 mM sodium citrate buffer for pH between 3 and 7, and in 100 mM Tris-HCl for higher pH values.

On the basis of the kinetic parameters of the *P. falciparum* proteinase for the fluorogenic substrates synthesized, the peptidic sequence Val-Leu-Gly-Lys has been selected as the most specific. As this *P. falciparum* parasitic proteinase was identified in free merozoites[3] and as a similar *P. berghei* enzyme was located in merozoites by immunofluorescence assays,[4] peptidic inhibitors and peptidyl drugs have been further synthesized with the aim to block the erythrocyte reinvasion step by merozoites.

Biological Testing. The effect of the pH on the hydrolysis of the substrate GlcA-Val-Leu-Gly-Lys-AEC by the *P. falciparum* proteinase is reported in Figure 1. This enzyme has a sharp activity between pH 6 and 9. All the hydrolysis assays and the in vitro experiments were therefore performed at the optimal pH 7.4 which furthermore corresponds to a physiological pH value. In Table I are given the K_m and the V_{rel} values characterizing the substrate affinity and the reaction velocity of the *P. falciparum* neutral proteinase, respectively. From the comparison of substrates S-3 and S-12, or S-5 and S-11, it appears that this enzyme releases the substituent of the carboxylic group of basic amino acid residues and preferentially when a lysine is in the P_1-position. The presence of an aromatic amino acid in P_2 or of an aromatic ring in P_3 drastically lowers the affinity of such substrates; see compounds S-14 and S-13, respectively. The replacement of the *N*-gluconoyl group by hydrophobic *tert*-butyloxy-

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Table II. Inhibition of the *P. falciparum* Proteinase Activity by the Peptidic Inhibitors Synthesized[a]

no.	inhibitors[c] P_5	P_4	P_3	P_2	P_1	P_1'		I_{50},[b] μM	K_i, mM
I-1	GlcA	Val	Leu	Gly	Lys	Sar-OH	a	320	0.25
I-2	GlcA	Val	Leu	Gly	Lys	NHC_2H_5	b	470	0.48
I-3	GlcA	Val	Leu	Gly	Arg	Sar-OH	c	1000	1.22
I-4	GlcA	Val	Leu	Gly	Lys	APD	a	1500	1.46
I-5	GlcA	Val	Leu	Gly	Lys	AB	a	1500	2.35
I-6	GlcA	Val	Leu	Sar	Arg	Sar-OH	c	2000	2.50
I-7	GlcA	Val	Leu	Gly	Lys	$N(C_2H_5)_2$	a	>2000	>2.50
I-8	GlcA	Val	Leu	Gly	Lys	Pro-OH	a	>2000	>2.50
I-9	GlcA	Val	Leu	Gly	Lys	OH	a	>2000	>2.50
I-10	GlcA	Val	Leu	Gly	Ala	NHC_2H_5		>2000	>2.50
I-11	Boc	Asp	Pro	Arg	Gly	NHC_2H_5	a	>2000	>2.50

[a] Bioassays are described in the Experimental Section. [b] I_{50}: concentration requested to get 50% inhibition of the hydrolysis of 0.19 mM GlcA-Val-Leu-Gly-Lys-AEC. [c] a, acetate; b, trifluoroacetate; c, hydrochloride; APD, 2-aminopropanediol; AB, (*R*)-2-amino-1-butanol.

carbonyl or benzyloxycarbonyl groups decreases markedly the affinity of the substrate for the enzyme and dramatically lowers the hydrosolubility of such substrates (**S-1a** and **S-1b** with regard to **S-1**). In the P_5-position, a hydrophylic gluconoyl group (substrate **S-1**) is also better than a positive charge on the N-terminal D-valine residue as in **S-1c**. For substrates **S-1** and **S-5**, the sequence Val-Leu in the P_4-P_3-position appears better than Ile-Val, although both dipeptides are highly hydrophobic. The high selectivity of the enzyme can be explained by a more favorable side-chain geometry, causing a better fit in the corresponding hydrophobic pockets of subsites 3 and 4. The length of the substrates has also an important role: tetrapeptide **S-7** is more specific than tripeptide **S-10**.

Among some 20 different substrates synthesized the best one is GlcA-Val-Leu-Gly-Lys-AEC. On this basis, peptidic inhibitors were synthesized by replacing the fluorescent amine present in the substrate by different alkylamines, amino alcohols known as potent inhibitors of *P. falciparum* parasites multiplication,[12] or a proline or a *N*-methylglycine (sarcosine) residue.

From Table II, peptides **I-1** and **I-2** appear as the most potent inhibitors of the series; peptides **I-9**, **I-10**, and **I-11** were used as controls. The inhibitory activity of peptide **I-1** can be explained by the presence of a nonhydrolysable secondary amide bond at the proteolytic cleavage site of the substrate. However, when the P_1' residue is too bulky, as is the case of proline residue (peptide **I-8**), no activity was observed. The same effect can be seen when the ethylamide group (**I-2**) is replaced by a diethylamide moiety (**I-7**), although this last compound has also a nonhydrolysable secondary amide bond.

Effects of the Pf 68 Proteinase Specific Inhibitors on Reinvasion. Reinvasion experiments with erythrocytes and synchronized *P. falciparum* mature schizonts in the presence of 10 mM GlcA-Val-Leu-Gly-Lys-NHC_2H_5 showed a markedly reduced number of new ring forms, while the number of merozoites loosely associated with the erythrocyte surface increased. Indeed, these merozoites were easily removed by a low-speed centrifugation (300*g* for 5 min), suggesting that their association only involved weak interactions. The inhibitory activity of the peptidylethylamide on any of the four strains tested was found to be independent of the experimental conditions: medium (RPMI or DMEM) and serum concentration (0, 5, or 10%). Experiments with different peptide concentrations (1.25–10 mM) and different initial parasitemia (1.25, 2.5, or 5%) evidenced that the inhibition of reinvasion was directly dependent upon the concentration of the peptidic inhibitors and inversely proportional to the initial parasitemia. Subsequent experiments demonstrated that the inhibition of red blood cell reinvasion was primarily related to the specific sequence Val-Leu-Gly-Lys (or Arg), as demonstrated by the lack of effect of peptides with unrelated sequences and of analogues of the proteinase substrates lacking the basic amino acid in the P_1-position (Table III). A correlation was observed between the in vitro inhibitory efficiency (IC_{50}) of the various peptides and their affinity toward the purified enzyme (K_i). Moreover, the ethylamine moiety released during incubation was not responsible for the inhibition of reinvasion and did not have any effect on the subsequent growth of new trophozoites, as 10 mM of ethylamine did not decrease the number of rings determined in the reinvasion after 6 h as well as the number of trophozoites determined after 24 h. Amino alcohols, such as 2-amino-1-butanol or 2-aminopropanol that impede the parasite lipid synthesis,[13] displayed an inhibitory activity when they are linked to the relevant peptide in the same way as the peptidylethylamide derivative (data not shown). The peptidic sequence Val-Leu-Gly-Lys is thus necessary to inhibit the reinvasion process.

Table III. Effects of Peptide Derivatives on the in Vitro Reinvasion of Red Blood Cells by *P. falciparum* Merozoites (IC_{50}) and on the Activity of the Purified Neutral Proteinase (K_i)

no.	inhibitors	IC_{50},[a] μM	K_i, μM
I-2	GlcA-Val-Leu-Gly-Lys-NHC_2H_5	900	480
I-7	GlcA-Val-Leu-Gly-Lys-$N(C_2H_5)_2$	>10000	>2500
I-9	GlcA-Val-Leu-Gly-Lys-OH	>10000	>2500
I-10	GlcA-Val-Leu-Gly-Ala-NHC_2H_5	>10000	>2500
I-12	Ac-Asp-Phe-Arg-Gly-NHC_2H_5	>10000	>2500

[a] The IC_{50} was determined from 6-h reinvasion experiments with an initial parasitemia of 5% segmenters (experiments in triplicate).

The C-terminal substituants of this peptide are of great importance in the modulation of the inhibitory activity of the compounds: GlcA-Val-Leu-Gly-Lys-OH (**I-9**) has no inhibitory activity, showing that the carboxylic group of the C-terminal basic amino acid must be substituted. It is noteworthy that the organic amine in P_1' position should be primary rather than secondary.

Peptide Derivatives of the Substrates for the Pf 68 Proteinase Inhibit the Invasion of Erythrocytes by Merozoites but Not the Merozoite Release. In order to discriminate between the two steps of the reinvasion process, i.e. the merozoite release from segmented infected red blood cells and the erythrocyte invasion by merozoites, peptide derivatives of the substrates for the Pf 68 proteinase were applied on a highly synchronized *P. falciparum* culture at the 44–48-h period of the erythrocytic life cycle which corresponds to the time T_0 of the experiment (Figures 2 and 3). Mature schizont infected red blood cells

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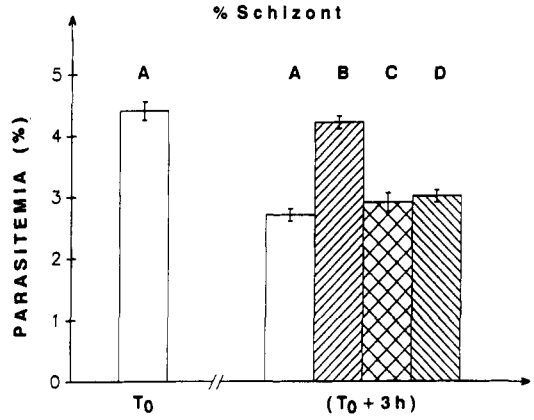


Figure 2. Inhibition of the merozoite release from schizonts. *P. falciparum* infected red blood cells at the segmented schizont stage (44–48 h range of ages, time T_0) were incubated during 3 h (T_0 + 3 h) without inhibitor (A) or with 1 mM leupeptin (B), 1 mM GlcA-Val-Leu-Gly-Lys-NHC_2H_5 (C), or 1 mM Ac-Asp-Phe-Arg-Gly-NHC_2H_5 (D). Effects of inhibitors on the merozoite release from schizonts were estimated by the percentage of resulting schizonts at T_0 + 3 h. Experiments were performed in triplicate and parasitemia was estimated by counting 5000 erythrocytes. Bars denote the standard error.

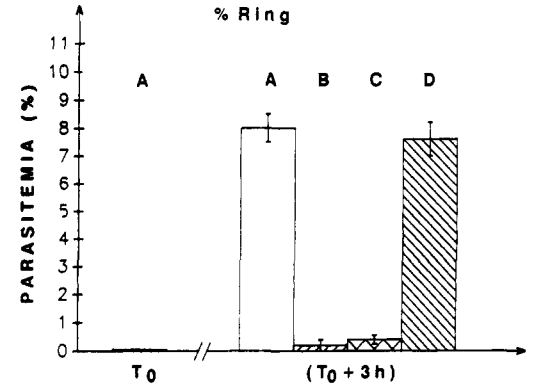


Figure 3. Inhibition of the erythrocyte invasion by the merozoites. *P. falciparum* infected red blood cells at the segmented schizont stage (44–48 h range of ages, time T_0) were incubated during 3 h (T_0 + 3 h) without inhibitor (A) or with 1 mM leupeptin (B), 1 mM GlcA-Val-Leu-Gly-Lys-NHC_2H_5 (C), or 1 mM Ac-Asp-Phe-Arg-Gly-NHC_2H_5 (D). Effects of inhibitors on the erythrocyte invasion were estimated by the percentage of new rings. Experiments were performed in triplicate and parasitemia was estimated by counting 5000 erythrocytes. Bars denote the standard error.

were incubated during 3 h in the presence of 1 mM leupeptin (B), 1 mM GlcA-Val-Leu-Gly-Lys-NHC_2H_5 (C), and 1 mM of a nonrelated peptide Ac-Asp-Phe-Arg-Gly-NHC_2H_5 (D). The reinvasion was compared to control cultures with no peptide (A) by counting unreleased schizonts and new rings.

After a 3-h incubation (T_0 + 3 h), GlcA-Val-Leu-Gly-Lys-NHC_2H_5 (Figure 2C) as well as the nonrelated peptide (Figure 2D) had no inhibitory effect on the merozoite release from segmenters since the number of schizonts was similar to that of the control (Figure 2A). In contrast, leupeptin inhibits drastically the merozoite release (Figure 2B). The invasion of the new erythrocytes by the merozoites occurred normally in presence of the nonrelated peptide (Figure 3D) as compared to the control (Figure 3A), but in the presence of GlcA-Val-Leu Gly-Lys-NHC_2H_5 the ring formation was completely inhibited (Figure 3C). The numbers of new rings were quite similar in the leupeptin series (Figure 3B) and in the peptide inhibitor of the Pf 68 proteinase series (Figure 3C). These results showed clearly that the low number of rings was due to a lack of the merozoite release in presence of leupeptin and to a specific inhibition of the invasion of erythrocytes by merozoites in presence of the peptide GlcA-Val-Leu-Gly-Lys-NHC_2H_5, without reduction of the merozoite release.

Discussion

The peptidic sequence Val-Leu-Gly-Lys (or Arg) is selective for the Pf 68 proteinase from the intraerythrocytic phase, as was the case for the *P. berghei* (Pb 68) and the *P. chabaudi* (Pc 68) proteinases.[2] As these *Plasmodium* enzymes have been characterized as thiol proteinases,[3] the selectivity of the substrates **S-1** and **S-2** has been assayed with regard to papain, another cystein proteinase active at pH 6.4. At 2 10^{-7} M papain concentration no proteolysis of these substrates was detected, indicating a high selectivity for the *P. falciparum* proteinase.

When GlcA-Val-Leu-Gly-Lys-NHC_2H_5 was added to synchronized cultures, just before the reinvasion step, a marked decrease in the formation of the new rings was observed while numerous merozoites were only weakly bound to the erythrocyte surface. These data suggest a specific inhibition of the reinvasion process. The inhibitory effect does not result from the release by proteolysis of the amino moiety such as ethylamine since free ethylamine did not inhibit erythrocyte reinvasion. The C-terminal substituents can however modulate the inhibitory activity of the compounds, as shown by the differences in in vitro efficiency of the various peptides synthesized. The peptidic sequence Val-Leu-Gly-Lys is essential for the inhibition of the in vitro reinvasion, as closely related sequences such as Val-Leu-Gly-Ala have no inhibitory effect. The results on the inhibition of the proteinase (k_i) or on the in vitro reinvasion process (IC_{50}) by Val-Leu-Gly-Lys derivatives strongly suggest an essential role of the Pf 68 proteinase during the reinvasion process. Such a role is also in agreement with the presence of the proteinase in free merozoites.[2,3]

The use of highly synchronized *P. falciparum* cultures allowed to discriminate the inhibitory effect of peptide derivatives on the two main steps of the reinvasion process: the merozoite release and the erythrocyte invasion per se. In contrast to leupeptin, Val-Leu-Gly-Lys derivatives did not inhibit the merozoite release from segmenters, but decrease the formation of new rings, suggesting that the Pf 68 proteinase acts during the erythrocyte invasion. However, a possible involvement of these specific peptidic inhibitors during the last step of the merozoite maturation, leading to noninvasive merozoites without affecting their release, cannot be excluded. During the schizogony, an increase of the permeability of the infected red blood cells was observed,[13] which could facilitate the entry of hydrophilic peptides inside the infected erythrocyte. This point will be cleared up by using macromolecular peptidic inhibitors in which Val-Leu-Gly-Lys derivatives will be coupled to the lysyl side chains of a polypeptidic carrier.

The inhibition of the *P. falciparum* proteinase and of the erythrocyte invasion require millimolar concentrations of the "inhibitors". In fact these substrate analogues were only used to identify the role of the proteinase. With such K_i they cannot be expected to be therapeutic agents. However, on the basis of these results, pseudopeptidic inhibitors which are now under investigation are expected to have a much higher efficienty since the sequence Val-Leu-Gly-Lys looks very specific and since a good correlation was observed between the protease inhibition and the erythrocyte invasion in in vitro experiments.

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Although the natural target of this parasite enzyme, specific for the substrate GlcA-Val-Leu-Gly-Lys-AEC, is not yet known, the fact that it is found among other *Plasmodium* species,[2] as well as among all the strains tested, suggests that its catalytic site is most probably conserved and therefore could provide a useful target for new chemotherapeutic approaches against malaria.

Experimental Section

All the amino acids used were of the L configuration unless otherwise stated. Amino acid derivatives were purchased from Novabiochem (Laufelfingen, Switzerland). 3-Amino-9-ethylcarbazole was from Sigma Chemical Co. (St. Louis, MO) and was recrystallized from methanol/hexane. All other reagents were of the best grade commercially available.

The monitoring of the syntheses was performed by thin-layer chromatography on silica gel plastic-backed plates (Merck 60 F-254) using the following eluting solvent systems: chloroform/methanol, 8:1 (v/v) (solvent A), chloroform/methanol/water, 80:30:2 (solvent B), chloroform/methanol/water, 6:6:1 (solvent C) chloroform/methanol, 4:1 (solvent D), chloroform/methanol, 12:1 (solvent E) and chloroform/methanol/water, 80:40:5 (solvent F). Compounds were successively visualized under UV light, by ninhydrin spray, and by *tert*-butylhypochlorite/starch iodine chromatic system or ammonium heptamolybdate in H_2SO_4 spray. Substrates and inhibitors were purified by HPLC with semipreparative-scale Gilson equipment with a variable-wavelength UV detector set at 216 nm and a 250 × 10 mm HIBAR column packed with 7-μm RP-18 Lichrosorb from Merck. The capacity factor k' of the compounds was determined by HPLC under analytical conditions from $k' = (t_R - t_0)/t_0$ where t_R is the retention time of the compound and t_0 is the time corresponding to the dead volume of the column.

Melting points, determined on a Leitz melting point apparatus, are uncorrected. Optical rotation measurements were performed on a Perkin-Elmer 141 polarimeter equipped with a thermostat. Infrared spectra were recorded on a Perkin-Elmer 457 spectrophotometer and UV absorption spectra were obtained on a Uvikon 860 spectrophotometer. Fluorescence emission or excitation spectra were recorded on a Perkin-Elmer LS 5 spectrofluorimeter standardized with quinine sulfate. Proton NMR spectra were recorded on a Bruker AM 300 spectrometer and are expressed as δ units (parts per million) relative to tetramethylsilane used as internal reference.

Cultures. *P. falciparum* was grown according to a modification of the original procedure of Trager and Jensen.[14] Four strains were used: FcR3/Gambia and Palo Alto (FUP)/Uganda (kindly provided by Prof. M. Hommel, Liverpool, U.K.), NF 54 (Dr. P. Druilhe, Paris, France), and FcB-1/Colombia (Dr. H. G. Heidrich, Martinsried bei München, FRG). The medium was either DMEM or RPMI 1640 containing 5–10% (v/v) human serum, 25 mM HEPES, supplemented with hypoxanthine, reduced glutathione (Sigma, St. Louis, MO, USA), and glucose according to Zolg et al.[15] and adjusted to pH 7.4 (complete medium).

The parasites were cultured in human type O^+ erythrocytes (Blood Bank, Poitiers, France) in culture flasks at a 3–4% hematocrit and gassed in a mixture of 3% CO_2, 6% O_2, and 91% N_2. For reinvasion experiments, 24-well microplates (Nunc, Rosklide, Denmark) with the candle jar method were used.

Synchronization. The parasites were synchronized by sequential combinaisons of schizont concentrations and sorbitol treatments.[16] Gelatin flotation[17] was used with the knob-forming strains NF 54 and FcB-1. Briefly, a suspension of parasitized erythrocytes (12.5% final hematocrit) in a v/v complete medium–gelatin mixture (Plasmagel, Roger Bellon, Neuilly, France) was allowed to sediment at 37 °C, and the supernatant containing trophozoites (usually 30–35-h old) was pelleted for 5 min at 300*g* and put back into culture during 12–15 h. Fresh erythrocytes were added when reinvasion began.

Isopycnic centrifugation with Percoll (Pharmacia, Les Ulis, France) was used with the knobless strains FcR-3/Gambian and FUP at the late schizont stage. A ca. 20% suspension of parasitized erythrocytes in complete medium was carefully layered on a 70% (v/v) isotonic Percoll solution and centrifuged for 15 min at 800*g*, washed in complete medium at 37 °C, spun down at 300*g* for 5 min, and immediately put back in culture with fresh erythrocytes. In both methods, reinvasion was stopped by destroying the remaining schizonts with 5% sorbitol (Calbiochem, La Jolla, CA) in distilled water. Reinvasion was not allowed to proceed for more than 4 h.

Enzyme Purification. Enzyme was purified as previously described.[3] Briefly, synchronized in vitro cultures of *P. falciparum* FcR-3 strain at the schizont stage were washed twice with cold isotonic solution: 0.15 M NaCl and 5 mM sodium phosphate buffer (PBS, pH 7.2). The parasites were released from red blood cells by saponin lysis (PBS containing 0.2% saponin w/v, 20 min, 37 °C) with gentle shaking. The free parasite fraction was washed four times with cold PBS (4 °C, 7 min, 600*g*). Pelleted parasites and free merozoites were lysed in 5 mM phosphate buffer, pH 7.2, for 30 min at 4 °C under swirling. The lysate was centrifuged for 30 min (100000*g* at 4 °C), and the pellet containing hemozoin, membranes, and erythrocytic and parasitic contaminants was discarded. The supernatant was concentrated by ultrafiltration on membrane cones (Centriflo CF 25, Amicon) and used as the crude enzyme extract. The enzyme was further purified by a two-step process involving chromatofocusing followed by gel filtration.[3] Chromatofocusing was carried out on a mono P 5/20 column (Pharmacia) using a linear pH gradient from 6.3 to 4.0. The active fraction was then concentrated to 50 μL on a CF 25 membrane cone and injected on a 30 × 1 cm Superose 12 gel filtration column (Pharmacia) eluted with 25 mM ammonium acetate. The fractions containing the enzyme which cleaves the substrate GlcA-Val-Leu-Gly-Lys-AEC were collected and the SDS-page analysis shows M_r = 68000. The enzyme will be referred as Pf 68 proteinase.

Enzyme Assays. The proteinase activity was monitored by a fluorimetric method. The enzyme extract (20 μL) was incubated for 30 min, at 37 °C, with 80 μL of peptidyl-AEC in 50 mM Tris-HCl buffer, pH 7.4. The AEC released by enzymatic hydrolysis was extracted with 1.0 mL ethyl acetate which solubilized selectively the free AEC and stopped the enzyme activity. The optimal excitation and emission wavelengths of free AEC are 370 and 430 nm, respectively. The substrate concentration for the kinetic studies varied between 0.5 and 2.0 mM. All the kinetic constants were determined according to the Lineweaver and Burk plots. Controls were done by incubating the peptidyl-AEC substrates for 30 min, at 37 °C, in the presence of 10 μg/mL trypsin. Under such conditions all the AEC was released from the substrate[9] and the fluorescence intensity of the ethyl acetate extract was taken as reference.

Inhibition of the Pf 68 Proteinase by Specific Peptide Derivatives. A solution of the fluorogenic substrate GlcA-Val-Leu-Gly-Lys-AEC (0.19 mM) was preincubated with the different peptidic inhibitors at concentrations between 0.005 and 1 mM in 50 mM Tris-HCl, pH 7.4, mixed with 20 μL of the enzymatic extract and incubated for 30 min, at 37 °C. The enzyme activity was determined as previously described. Controls were done for each inhibitor under the same conditions but in the absence of protease. The I_{50} values (inhibitor concentration corresponding to 50% inhibition of the proteinase activity) were determined from the plot of the proteinase activity versus the inhibitor concentration.

Erythrocyte Reinvasion Inhibition. Healthy red blood cells were washed twice with RPMI medium and resuspended at 5% (v/v) hematocrit in complete culture medium containing different concentrations of peptide derivatives. Mature parasites (schizonts 44–48 h range of ages) were collected by Plasmagel procedure from a 4-h synchronized culture and added to healthy erythrocytes to reach 4–5% parasitemia (time T_0). Usually, all the peptide derivatives were tested in 24-well microplates at a 5% hematocrit in culture medium with 5% serum after a 4–6-h incubation in a candle jar. Then the cells were pelleted in a Beckman microfuge and 1000 cells were blind counted on Giemsa-stained smears. For discrimination between the merozoite release from segmented

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Scheme I. Synthesis of the Fluorogenic Substrate GlcA-Val-Leu-Gly-Lys-AEC

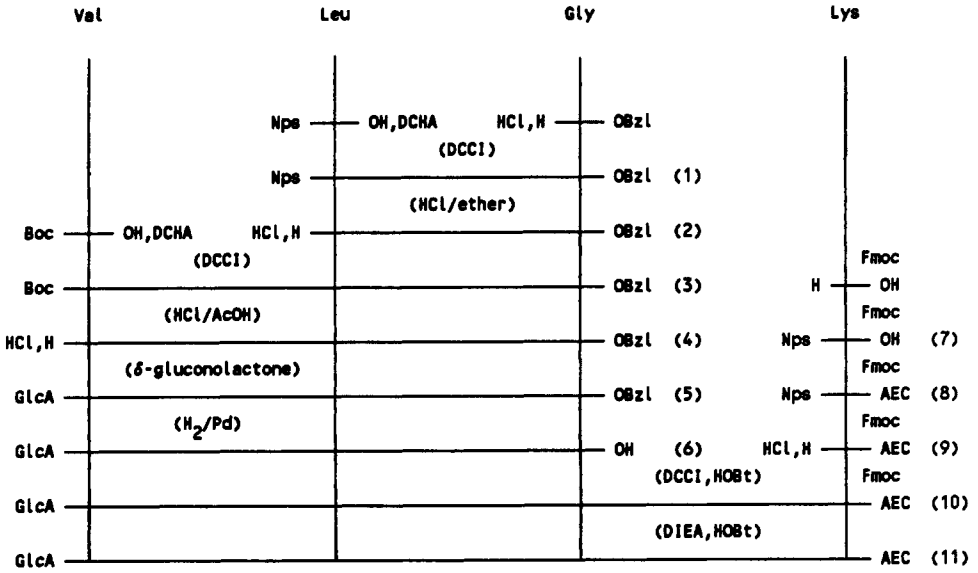


Table IV. Physical Data of the Synthesized Fluorogenic Substrates

no.	substrates P_5	P_4	P_3	P_2	P_1	P_1'	M_w	mp, °C	$[\alpha]^{25}_{546}$ (c = 1, MeOH), deg	k'[a]
S-1	GlcA	Val	Leu	Gly	Lys	AEC	821.5	141–143	−32.5	5.16 (a)
S-1a	Boc	Val	Leu	Gly	Lys	AEC	743.5	186–189	−26.9 (DMF)	2.75 (b)
S-1b	Z	Val	Leu	Gly	Lys	AEC	777.5	193–196	−0.2 (DMF)	2.97 (b)
S-1c	H	D-Val	Leu	Gly	Lys	AEC	756.5	150–153	−78.1	3.61 (b)
S-2	GlcA	Val	Leu	Gly	Arg	AEC	849.5	159–160	−22.5	3.66 (a)
S-3		GlcA	Ser	Gly	Lys	AEC	696.5	123–127	−24.6	3.01 (c)
S-3a		Boc	Ser	Gly	Lys	AEC	618.5	143–148	−48.2 (H_2O)	-
S-4	GlcA	Thr	Leu	Gly	Lys	AEC	823.5	109–113	−26.5	2.16 (a)
S-5	GlcA	Ile	Val	Gly	Lys	AEC	821.5	31–35	−18.6	1.62 (d)
S-6	GlcA	Ser	Leu	Gly	Lys	AEC	809.5	146–150	−28.2	3.94 (a)
S-7	GlcA	Val	Thr	Gly	Lys	AEC	809.5	147–150	−10.0	6.33 (c)
S-8	GlcA	Val	Leu	Sar	Lys	AEC	835.5	hygr	+4.1	4.75 (a)
S-9	GlcA	Ala	Ala	Ala	Lys	AEC	765.5	>250 dec	−29.0	9.22 (e)
S-10		GlcA	Thr	Gly	Lys	AEC	710.5	105–109	−20.0	4.22 (c)
S-11	GlcA	Ile	Val	Gly	Arg	AEC	849.5	131–134	+87.4	1.64 (d)
S-12		GlcA	Ser	Gly	Arg	AEC	724.5	168–172	−24.1	3.34 (c)
S-12a		Boc	Ser	Gly	Arg	AEC	646.5	138–141	−31.3	-
S-12b		GlcA	Ser(Bzl)	Gly	Arg	AEC	814.5	141–145	−27.7	22.4 (c)
S-13		GlcA	Ser(Bzl)	Gly	Lys	AEC	786.5	101–105	−28.0	19.2 (c)
S-14	GlcA	Val	Gly	Leu	Lys	AEC	821.5	208–211	−45.9	34.6 (e)
S-15	GlcA	Ala	Thr	Gly	Lys	AEC	781.5	104–107	−15.3	4.52 (c)

[a] Retention times of the substrates, determined by HPLC, are expressed in terms of their capacity factors k' defined in the Experimental Section. Solvent systems were CH_3CN/H_2O 35:65 with 0.1% TFA (a), CH_3CN/H_2O 70:30 with 10 mM ammonium acetate (b), CH_3CN/H_2O 30/70 with 0.1% TFA (c), CH_3CN/H_2O 60:40 with 10 mM ammonium acetate (d), CH_3CN/H_2O 25/75 with 0.1% TFA (e).

schizonts and the erythrocyte invasion by merozoites, aliquots were incubated during 3 h with 1 mM GlcA-Val-Leu-Gly-Lys-NHC_2H_5, or 1 mM leupeptin, or 1 mM nonrelated peptide. Each point was performed in triplicate, and parasitemia as determined by counting 5000 red blood cells from Giemsa-stained smears. The percentage of inhibition was calculated by $p = 100 - (R/R_0 \times 100)$, where R stands for the number of new ring-infected red blood cells in the treated cultures, and R_0 for the number of new ring-infected red blood cells in the corresponding control cultures. The number of remaining schizonts and segmenters as well as the number of morphologically degenerative parasites were also determined.

Peptide Synthesis. The synthesis strategy, described in details for GlcA-Val-Leu-Gly-Lys-AEC, is shown in Scheme I, and the physicochemical properties of the various fluorogenic peptidic substrates, synthesized in a similar way, are reported in Table IV. The reversible peptidic inhibitors were prepared by using the same strategy: a racemization-free fragment coupling between GlcA-Val-Leu-Gly-OH and H-Lys(Z)-R, where R is either an aliphatic amine (ethylamine or diethylamine), proline, *N*-methylglycine, or the amino alcohols 2-methyl-1,3-propanediol or 2-amino-1-butanol; their physicochemical properties are reported in Table V.

Table V. Physical Data of the Reversible Inhibitors Synthesized

no.	inhibitors[a] P_2 P_1 P_1'	M_w	mp, °C	k'[b]
I-1	R-Gly-Lys-Sar-OH·HCl	700.5	96–99	0.9 (a)
I-2	R-Gly-Lys-NHC_2H_5·HCl	656.5	53–56	2.8 (b)
I-3	R-Gly-Arg-Sar-OH·TFA	806.4	hygr	3.7 (c)
I-4	R-Gly-Lys-APD·HCl	702.5	hygr	3.6 (b)
I-5	R-Gly-Lys-AB·HCl	700.5	109–113	5.7 (b)
I-6	R-Sar-Arg-Sar-OH·TFA	820.4	hygr	4.8 (c)
I-7	R-Gly-Lys-$N(C_2H_5)_2$·HCl	684.5	51–54	2.6 (c)
I-8	R-Gly-Lys-Pro-OH·HCl	726.5	129–133	1.3 (c)
I-9	R-Gly-Lys-OH·HCl	629.5	47–51	1.0 (c)
I-10	R-Gly-Ala-NHC_2H_5	563.3	98–102	1.4 (c)

[a] R = GlcA-Val-Leu, APD = 2-aminopropanediol, AB = (*R*)-2-amino-1-butanol. [b] Retention times of the inhibitors, determined by HPLC, are expressed in terms of their capacity factors k'. Solvent systems were CH_3CN/H_2O 20:80 with 10 mM ammonium acetate (a), CH_3CN/H_2O 30:70 with 10 mM ammonium acetate (b), CH_3CN/H_2O 20:80 with 0.1% TFA (c).

Nps-Leu-Gly-OBzl (1). To a solution of glycine benzyl ester hydrochloride (4.03 g, 20 mmol) in dry ethyl acetate (200 mL)

was added Nps-Leu-OH·DCHA (9.30 g, 20 mmol). The reaction mixture was cooled down to 0 °C and DCC (4.53 g, 22 mmol) was added under stirring. Stirring was continued at 0 °C for 2 h and at room temperature for 12 h. Dicyclohexylurea and HCl·DCHA were removed by filtration, and the filtrate was washed successively with saturated $NaHCO_3$, water, 5% $KHSO_4$, and water, dried (Na_2SO_4), and evaporated in vacuo to yield 7.78 g (90%) of an oil: R_f 0.85 (solvent A).

H-Leu-Gly-OBzl·HCl (2). The dipeptide Nps-Leu-Gly-OBzl (1; 7.78 g, 18 mmol) was dissolved in a 1:1 acetone/ether mixture (150 mL). A 12-mL portion of 4.3 N HCl/ether was added and the mixture was stirred for 20 min. The solution was precipitated in hexane and the residue was triturated several times in hexane. The hygroscopic precipitate was dried in vacuo to yield 5.2 g (92%): R_f 0.15 (solvent A); argentimetric titration 99%.

Boc-Val-Leu-Gly-OBzl (3). To a solution of 2 (3.78 g, 12 mmol) in chloroform (110 mL) was added Boc-Val-OH·DCHA (4.92 g, 12 mmol). The reaction mixture was cooled down to 0 °C and DCC (2.72 g, 13.2 mmol) was added under stirring. The stirring was continued at 0 °C for 2 h and at room temperature for 12 h. The solvent was removed by evaporation in vacuo and ethyl acetate (100 mL) was added. The precipitate of DCU and HCl·DCHA was removed by filtration, and the filtrate was washed successively with saturated $NaHCO_3$, water, 5% $KHSO_4$, and water, and then dried, concentrated, and precipitated in cold hexane to yield 5.06 g (88%): mp 66–68 °C; $[\alpha]^{25}_{546}$ −51.9° (*c* = 1, $CHCl_3$); R_f 0.78 (solvent A).

H-Val-Leu-Gly-OBzl·HCl (4). A solution of 3 (3.35 g, 7.0 mmol) in 1 N HCl/AcOH (35 mL) was stirred for 20 min at room temperature. The excess of reagent was removed by evaporation under reduced pressure and the residue was dissolved in methylene chloride (20 mL) and precipitated in 1:1 ether/petroleum ether. The precipitate was washed three times with ethyl ether, filtrated, and dried to yield 2.7 g (95%): mp 105–107 °C; $[\alpha]^{25}_{546}$ −30.2° (*c* = 1, $CHCl_3$); R_f 0.80 (solvent B); argentimetric titration 99%.

GlcA-Val-Leu-Gly-OBzl (5). To a solution of 4 (2.70 g, 6.5 mmol) in 3:1 DMF/DMSO (8 ml) were added δ-gluconolactone (3.47 g, 19.5 mmol) and triethylamine (2.73 mL, 19.5 mmol). The mixture was stirred for 48 h at 50 °C and then cooled and filtrated. The filtrate was concentrated in vacuo and the residue was purified by preparative chromatography on a silica gel column eluted with the solvent system B to yield 2.20 g (60%) after precipitation in 1:1 ether/petroleum ether: mp 103–105 °C; $[\alpha]^{25}_{546}$ −24.4° (*c* = 1, CH_3OH); R_f 0.62 (solvent B).

GlcA-Val-Leu-Gly-OH (6). Tripeptide ester 5 (1.34 g, 2.4 mmol) was hydrogenolyzed over Pd/C (10% Pd) (0.3 g) in 3:1 methanol/water (20 mL) for 6 h at room temperature. The product was isolated, after removal of the catalyst by filtration, in 95% yield (1.07 g): mp 88–90 °C; $[\alpha]^{25}_{546}$ =15.7° (*c* = 1, CH_3OH); R_f 0.52 (solvent C).

Nps-Lys(Fmoc)-OH (7). N^{ϵ}-Fmoc-lysine (2.58 g, 7.0 mmol) was dissolved in a mixture 70:18:7 of dioxane/water/1 N NaOH and acylated by 2-nitrobenzenesulfenyl chloride (1.33 g, 7.0 mmol). The pH of the solution was kept at 7.5 by using a pH-stat and addition of 1 N NaOH (7 mL). The insoluble material was filtered off and the filtrate was diluted with water (30 mL) and acidified to pH 3.0 with 0.1 N H_2SO_4. The product was rapidly extracted with ethyl acetate. The organic phase was washed with cold water, dried (Na_2SO_4), concentrated, and precipitated in cyclohexane to yield 3.32 g (91%): mp 70–72 °C; $[\alpha]^{25}_{546}$ −13.0° (*c* = 1, CH_3OH); R_f 0.63 (solvent D).

Nps-Lys(Fmoc)-AEC (8). To a solution of 7 (2.32 g, 4.45 mmol), 3-amino-9-ethylcarbazole (0.93 g, 4.45 mmol), and HOBt·H_2O (0.68 g, 4.45 mmol) in freshly distilled DMF (10 mL) was added DCC (1.00 g, 0.49 mmol) at 0 °C under stirring and under nitrogen atmosphere. Stirring was continued at 0 °C for 2 h and at room temperature for 12 h. DCU was removed by filtration and the filtrate was evaporated to dryness. The crude product was purified by silica gel column chromatography with the solvent system E to yield 2.57 g (80%): mp 120–122 °C; $[\alpha]^{25}_{546}$ −11.1° (*c* = 1, DMF); R_f 0.88 (solvent E).

H-Lys(Fmoc)-AEC·HCl (9). To a solution of 8 (1.43 g, 2.0 mmol) in chloroform (40 mL) was added 1.16 mL of 4.3 N HCl/ether and the mixture was stirred for 20 min. The solution was precipitated in dry ethyl ether and the precipitate was washed with ether and dried over KOH pellets to give 1.14 g (95%) of 9: mp 175–176 °C; $[\alpha]^{25}_{546}$ 38.1° (*c* = 1, DMF); R_f 0.84 (solvent F); argentimetric titration 99.3%.

GlcA-Val-Leu-Gly-Lys(Fmoc)-AEC (10). To a solution of 9 (0.20 g, 0.33 mmol), GlcA-Val-Leu-Gly-OH (6; 0.15 g, 0.33 mmol), triethylamine (45 μL, 0.32 mmol), and HOBt·H_2O (0.05 g, 0.33 mmol) in freshly distilled DMF (2 mL) was added DCC (0.075 g, 0.36 mmol) at 0 °C under stirring. Stirring was continued at 0 °C for 2 h and at room temperature for 12 h. DCU and HCl·TEA were removed by filtration, and the filtrate was evaporated to dryness. The crude product was purified by silica gel column chromatography with chloroform/methanol/water (80:40:5): yield 0.20 g (60%); mp 161–162 °C; $[\alpha]^{25}_{546}$ −33.2° (*c* = 1, DMF); R_f 0.92 (solvent F).

GlcA-Val-Leu-Gly-Lys-AEC·HCl (11). The N^{ϵ}-Fmoc protecting group of 10 (0.15 g, 0.15 mmol) was removed in a 10% solution of diethylamine in DMF (3 mL) after 20 min of stirring. The excess of reagent was completely evaporated in vacuo and 4.3 N HCl/ether (35 μL) was added. The product was then precipitated by addition of dry ether and filtered. The precipitate was dried in vacuo under KOH pellets to give 0.11 g (90%). The compound was finally purified by preparative-scale reversed-phase HPLC with a 7 μM RP 18 Lichrosorb packing eluted with water/acetonitrile (65:35) containing 0.1% TFA: mp 141–142 °C; $[\alpha]^{25}_{546}$ −32.7° (*c* = 1, CH_3OH); R_f 0.17 (solvent F); NMR (DMSO-d_6, 300 MHz) δ 0.84 (CH_3, Val and Leu), 1.30 (CH_3, AEC), 1.41 (γ-CH_2, Lys), 1.57 (δ-CH_2, Lys), 1.65–1.81 (β-CH_2, Lys), 1.67 (β-CH_2, Leu), 2.07 (β-CH, Val), 2.79 (ε-CH_2, Lys), 3.41–3.58 (CH_2-6, GlcA), 3.50 (CH-5, GlcA), 3.58 (CH-4, GlcA), 3.75 (α-CH_2, Gly), 3.97 (CH-3, GlcA), 4.11 (CH-2, GlcA), 4.16 (α-CH, Val), 4.26 (α-CH, Lys), 4.36 (OH-6, GlcA), 4.41 (α-CH, Leu), 4.43 (CH_2, AEC), 4.52 (OH-3, GlcA), 4.58 (OH-4, and OH-5, GlcA), 5.69 (OH-2, GlcA), 7.16 (CH-6, AEC), 7.45 (CH-7, AEC), 7.55 (α-NH, Val), 7.56 (CH-1, AEC), 7.58 (CH-8, AEC), 7.60 (CH-2, AEC), 7.81 (ε-NH_2, Lys), 8.00 (α-NH, Leu), 8.09 (α-NH, Lys), 8.16 (α-NH, Gly), 8.45 (CH-4, AEC), 9.92 (NH, AEC).

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Registry No. 1, 65895-26-3; 2, 60079-62-1; 3, 37783-46-3; 4, 121459-61-8; 5, 121459-62-9; 6, 121459-63-0; 7, 135523-68-1; 8, 135523-69-2; 9, 135523-70-5; 10, 135523-71-6; 11·HCl, 135635-59-5; 11 (free base), 121613-22-7; I-1·HCl, 135635-60-8; I-1 (free base), 121459-59-4; I-2·HCl, 121522-70-1; I-2 (free base), 121459-49-2; I-3·TFA, 135635-61-9; I-3 (free base), 121613-43-2; I-4·HCl, 135635-62-0; I-4 (free base), 121459-57-2; I-5·HCl, 135680-92-1; I-5 (free base), 135635-64-2; I-6·TFA, 135523-79-4; I-6 (free base), 135523-78-3; I-7·HCl, 121522-71-2; I-7 (free base), 121459-50-5; I-8·HCl, 135523-80-7; I-8 (free base), 135635-65-3; I-9·HCl, 135635-63-1; I-9 (free base), 121613-47-6; I-10 (free base), 135523-81-8; I-11 (free base), 135523-82-9; I-12 (free base), 135523-83-0; S-1a, 121613-28-3; S-1b, 121613-32-9; S-1c, 121613-33-0; S-2, 134054-53-8; S-3, 121613-24-9; S-3a, 135523-72-7; S-4, 121613-25-0; S-5, 121613-26-1; S-6, 121613-27-2; S-7, 121613-29-4; S-8, 121613-30-7; S-9, 135523-73-8; S-10, 135523-74-9; S-11, 121613-31-8; S-12, 121613-35-2; S-12a, 135523-75-0; S-12b, 135523-76-1; S-13, 121613-36-3; S-14, 121613-34-1; S-15, 135523-77-2; Nps-Cl, 7669-54-7; Nps-Leu-OH·DCHA, 7675-50-5; H-Gly-OBzl·HCl, 2462-31-9; Boc-Val-OH·DCHA, 16944-17-5; H-Lys-(FMOC)-OH, 84624-28-2; H-Lys(Z)-NHEt, 121459-65-2; H-Lys-(Z)-NEt_2, 135523-85-2; H-Lys(Z)-Pro-OH, 42001-60-5; H-Lys-(Z)-Sar-OH, 135523-86-3; H-Lys(Z)-APD, 135523-87-4; H-Lys-(Z)-AB, 135523-88-5; δ-gluconolactone, 90-80-2; 3-amino-9-ethylcarbazole, 132-32-1; plasmodium falciparum proteinase, 9001-92-7.

Supplementary Material Available: Amino acid analysis for substrates and inhibitors synthesized (1 page). Ordering information is available on any current masthead page.