High Antiplasmodial Activity of Novel Plasmepsins I and II Inhibitors

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The aim of this study was to develop new antiplasmodial compounds acting through distinct mechanisms during both the liver and the blood stages of the parasite life cycle. Compounds were designed on the basis of the "double-drug" approach: primaquine, which has been linked to statine-based inhibitors of plasmepsins (PLMs), the plasmodial aspartic proteases involved in degradation of hemeoglobin. The compounds were tested in vitro for anti-PLM I/PLM II activities and against chloroquine-sensitive (D10) and chloroquine-resistant (W2) strains of *P. falciparum*. An antiplasmodial activity (IC_{50}) as low as 0.1 μM was obtained, an excellent improvement in comparison with inhibitors previously reported ($IC_{50} = 2-20$ μM). The killing activity was equally directed against both *P. falciparum* strains and was correlated to lipophilicity (calculated as ALogP), for all compounds but one (**9**). All compounds inhibited PLM I and PLM II in the nanomolar range ($K_i = 1-700$ nM). The most promising compounds (**2**, **6**, **10**) were not cytotoxic against human fibroblasts at 100 μM and were highly selective for PLMs vs human cathepsin D.

Introduction

Malaria, with AIDS and tuberculosis, is one of the three major communicable diseases linked to poverty. The unavailability of a vaccine and the spread and intensification of drug resistance over the past 15−20 years have led to a dramatic decline in the efficacy of the most affordable antimalarial drugs.[1−3] In order to circumvent that situation, medications based on the association of two substances acting against the parasite have been developed.

The basis of this study was to develop new compounds acting through distinct mechanisms during both the liver stage and the blood stage of the life cycle of the parasite. Compounds were designed on the basis of the "double-drug" approach: primaquine (PQ[a]), active against the gametocytes and hypnozoites of the relapsing malaria parasites *P. vivax* and *P. ovale* and which has been linked to statine-based inhibitors of plasmepsins (PLMs), the aspartic proteases involved in degradation of hemoglobin during the blood stage of the parasite.[4]

The therapy with PQ has been reconsidered after the observation that PQ is a safe and effective agent for the prophylaxis of malaria due to both *P. falciparum* and *P. vivax*.[5] Furthermore, the use of the prodrug Val-Leu-Lys-PQ reduced toxicity and increased the activity of the drug.[6]

Several inhibitors of PLM II have already been designed by replacement of the scissile peptide bond with a transition state analogue,[7,8] including PS777621 characterized by a statine-based core (Figure 1).[9] A drawback for the development of substrate-based PLM II inhibitors was that while enzyme inhibition occurred at the nanomolar level, effectiveness of the inhibitors in killing the parasite was limited ($IC_{50} = 2-20$ μM).[9−11] Differences of this kind were attributed to low bioavailability probably because most of these inhibitors are derived from peptides. However, in view of recent publications, the low potency against cultured parasites may also be explained by the plasmepsins' redundancy,[12,13] which implies that blockade of this enzyme family may not be lethal.[14]

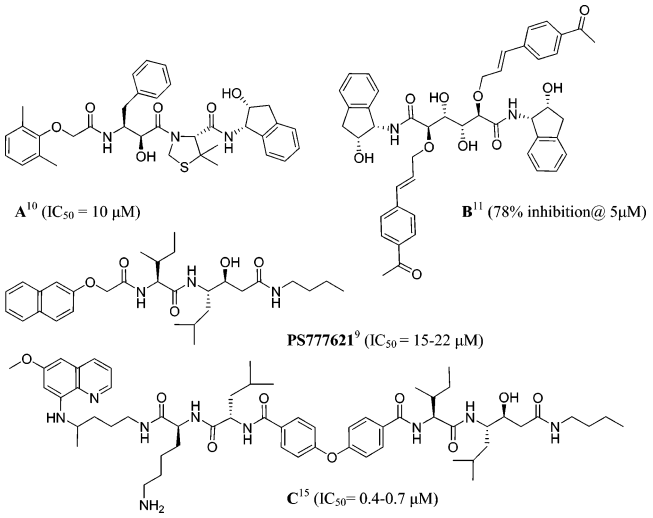

Figure 1. *P. falciparum* growth inhibition by three plasmepsin inhibitors encompassing a norstatine (**A**), a 1,2-dihydroxyethylene (**B**), and a statine (PS777621) motif. Compound **C** represents the most potent inhibitor in our previous series.

The "double-drug" strategy might help to improve cell permeability and pharmacological activity while reducing dose and side effects. Following this strategy, we published the antiplasmodial activity of a series of compounds that have a statine moiety bound to Leu-Lys-PQ residue by means of different linkers.[15] The best IC_{50} value for the inhibition of *P. falciparum* was 0.4 μM, thus achieving a remarkable improvement with respect to other inhibitors of PLM II.[8−11] We also

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[a] Abbreviations: PLM, plasmepsin; PQ, primaquine; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria Bertani medium; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; CQ, chloroquine; RPMI, Roswell Park Memorial Institute.

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demonstrated that the removal of the Lys-PQ moiety led to a drastic reduction of the antiparasitic activity.

In this paper we report the synthesis and the biological evaluation of nine new statine-based PLMs inhibitors (Table 1). Our efforts were mainly directed in improving the killing on the parasite during the intraerythrocytic stage. All synthesized compounds presented the statine moiety unchanged, while modifications were made to the Leu-Lys-PQ unit (Table 1). In particular, the first variations were directed toward the dipeptide Leu-Lys by removing Lys (**2**) or both amino acids (**3**). In compounds **4**−**8** the Leu-Lys dipeptide has been modified by introducing Ala in place of the Lys and/or Leu residue or by protecting the N$^{\epsilon}$ of Lys with a Boc group. In compounds **9** and **10** the PQ residue has been substituted with a propyl ester and the N$^{\epsilon}$ of Lys was unsubstituted or protected with a Boc group.

The compounds have been tested in vitro against recombinant PLMs I and II, and against D10 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *P. falciparum*, using compound **1** (representing the previously described PS777621) as the reference molecule. The selectivity over human cathepsin D, which shares a high homology with PLMs, and cell toxicity on mammalian cells were also investigated for the most promising compounds.

Results

Chemistry. In view of the high costs of commercially available statine, a gram-scale synthesis of *N*-Boc-statine has been developed in order to obtain compounds **1**−**10 (**Schemes 1 and 2). Stereoselective addition of the zinc enolate of ethylacetate[16] to *N*-Boc-leucinal[17] resulted in *N*-Boc-statine ethyl ester **11**, which after hydrolysis and coupling with butylamine followed by Boc removal and coupling with BocIleOH gave intermediate **12**, easily purified by crystallization. This synthesis required only one chromatographic purification step (after enolate addition), and the overall yields from Boc-leucine were 15%. After deprotection of **12** to give amine **13** and coupling with the monobenzyl ester of 4,4′-oxybisbenzoic acid **14**, obtained by selective monobenzylation of 4,4′-oxybisbenzoic acid, compound **15**, the statine-containing portion of inhibitors **3**−**8**, was obtained (Scheme 1). The PQ-containing portions, **18**−**22**, of inhibitors **4**−**8** were obtained by reaction of PQ diphosphate with Boc-leucine or Boc-alanine using standard coupling conditions to give compounds **16** and **17**, which were coupled to Cbz-leucine or Cbz-alanine to give PQ-containing fragments **18**−**20**. Lysine-containing fragments **21** and **22** were obtained by coupling with 4′-*N*-(*N*$^{\epsilon}$-Boc-lysyl)PQ[6] (Scheme 1).

The PQ-containing fragments **18**−**20** and **22** and the statine-containing fragment **15** were simultaneously deprotected and coupled to afford final compounds **5**−**8**. Compound **3** was obtained by coupling of **15** with PQ and compound **4** by coupling of **15** with **21** followed by deprotection of the lysine N$^{\epsilon}$ Boc group and formation of the diphosphate salt (Scheme 1). For the synthesis of **2** a slightly different synthetic approach was undertaken (Scheme 2). The monobenzyl ester of 4,4′-oxybis(benzoic acid) **14** was first coupled to 4′-*N*-(Boc-leucyl)-PQ **17** and subsequently coupled with the statine-containing fragment **13**.

The synthesis of compounds **1**, **9**, and **10**, not containing PQ, was carried out as described in Scheme 2. Compound **1** was prepared by coupling 2-naphthoxyacetic acid to **13**. Fully protected lysine derivative **25** was prepared by N$^{\epsilon}$ Boc protection of Cbz-lysine allyl ester,[18] which was coupled with Cbz-leucine to give compound **26**, followed by coupling with the monobenzyl ester of 4,4′-oxybis(benzoic acid) **14** to give dipeptide intermediate **27**, which was coupled to statine salt **13** to give compound **10**. Compound **9** was prepared by N$^{\epsilon}$ Boc deprotection of **10**.

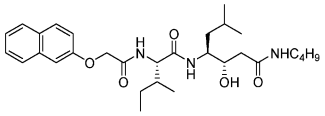
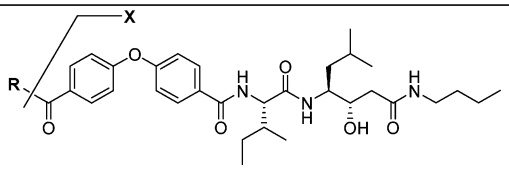
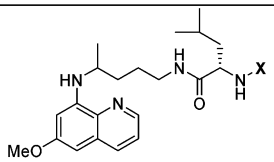
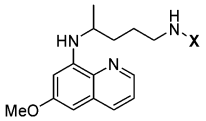
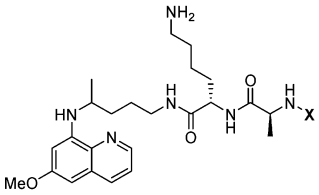
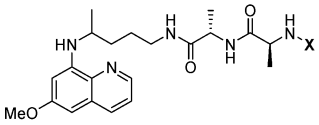
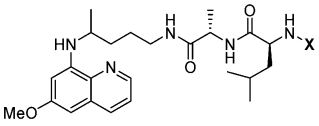
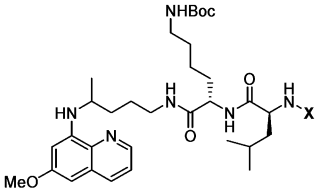
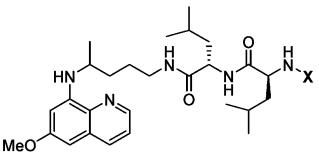
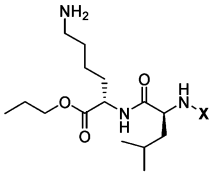
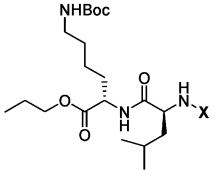
Effect of Compounds on PLM I and PLM II Activities. All compounds inhibited both PLM I and PLM II in a concentration-dependent fashion (Table 1). PLM I inhibition was improved in comparison to **1** for all the compounds with the exception of compound **2**. In particular, compound **2,** deprived of Ala, and compound **7**, where the N$^{\epsilon}$ of Lys is Boc-protected, possessed the lowest affinity for PLM I (K_i of 765 and 230 nM, respectively). For compounds **2**−**6**, K_i values for PLM II inhibition ranged between 4 and 26 nM, which represents an improvement with respect to **1**, while for **7** and **8** the affinity decreased. Compounds **9** and **10** showed similar values for inhibition of PLMs I and II, (K_i = 10−17 nM). Compound **5** deserves particular attention in view of the highest inhibition against both PLMs (1.0 and 3.9 nM for PLM I and PLM II, respectively), demonstrating a remarkable improvement toward PLM I (>700-fold vs **1**).

As assessed by SDS−PAGE (Figure 2), compounds **2**, **6**, and **10**, the best inhibitors of the parasite growth (Table 1), were able to prevent the human hemoglobin cleavage induced by PLM II, a result that further supports the possibility that these molecules are able to inhibit the enzyme when hemoglobin is the substrate for *P. falciparum* PLMs.

Effect of Compounds on *P. falciparum* Growth. All PQ-statine compounds inhibited parasite growth in a dose-dependent manner, and IC_{50} values against both strains (0.2−4 μM) are significantly below that of the reference compound **1** (15−23 μM), confirming that the "double-drug" approach represents a good strategy for improving the effectiveness of PLM inhibitors to starve and kill the parasite. By comparison of compounds **4**−**8** characterized by a dipeptide linking PQ to the statine portion of the molecule, compound **6** with the Leu-Ala dipeptide was found to be the most active compound against both D10 chloroquine-sensitive and W2 chloroquine-resistant strains of *P. falciparum* (0.2 and 0.3 μM, respectively). Modifications at the Leu-Ala dipeptide decreased the antiplasmodial activity (**4**, **5**, **7**, **8**). While compound **2** showed a lower activity against the D10 strain than compound **6** and while the effect on W2 was comparable, compound **3** showed a sharp decrease in activity against both strains. In our previous study[15] we demonstrated that the removal of aminoquinoline led to a dramatic loss of inhibitory activity, while, as shown here, compound **10**, where PQ is replaced by a propyl ester group and the amino group of Lys is protected, still maintains a very high inhibitory activity (IC_{50} = 0.1 μM). For compound **9**, where the amino group of Lys is not Boc-protected, the killing effect worsened (IC_{50} = 3.8−4.5 μM).

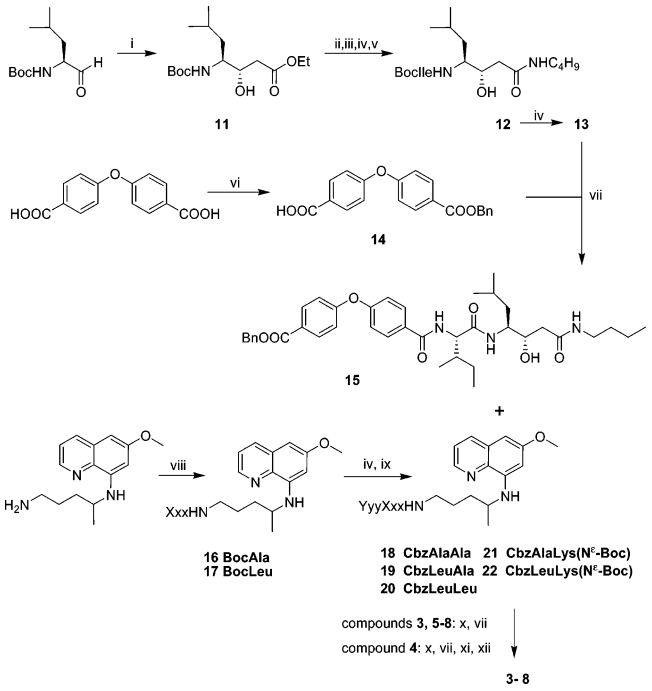
Molecular Modeling. The 3D structural models of compound **6**, one of the most promising compounds, were constructed from pepstatin A using the X-ray crystal coordinates of pepstatin A in complex with plasmepsin II (PDB entry 1XDH) as a starting point. Figure 3 shows a superimposition of the conformers of **6** with pepstatin A, while the conformers of **6**, without pepstatin A, in complex with PLM II are shown in Figure 4. Molecular modeling shows that the side chains of Ile and Sta and the *N*-butylammido group are easily superimposed with pepstatin A (Figure 3) and are nicely accommodated within the active site (Figure 4). The conformational freedom of the 4,4′-oxybisbenzoic ring system allows the LeuAla-PQ part of the molecule to assume several conformations in which PQ is partially or totally exposed to the solvent (Figure 4). PQ does

Table 1. Enzyme Inhibition, Antiplasmodial Activity, and Mammalian Cell Toxicity of Inhibitors[a]

Compd.	Structure	PLM I Ki (nM)	PLM II Ki (nM)	D10 IC_{50} (μM)	W2 IC_{50} (μM)	Cat D	Mamm. Cell Tox.
1		727 ± 57	58.9 ± 4.2	22.7 ± 4.04	15.4 ± 0.82		
	 **R**						
2		765 ± 56	12.1 ± 1.1	0.7 ± 0.11	0.5 ± 0.23	30% @ 2 μM	N.D. @ 100μM
3		81 ± 7	11.2 ± 0.8	2.2 ± 0.69	2.0 ± 0.25		
4		33 ± 5	10.6 ± 1.9	0.8 ± 0.03	1.4 ± 0.34		
5		1 ± 0.5	3.9 ± 0.41	1.3 ± 0.17	1.2 ± 0.52		
6		74 ± 11	26.3 ± 4.9	0.2 ± 0.12	0.3 ± 0.13	50% @ 5μM	15% @ 100μM
7		230 ± 30	403 ± 20	4.05 ± 2.29	3.5 ± 1.0		
8		58 ± 4	103 ± 4.4	3.6 ± 2.13	1.7 ± 1.7		
9		11 ± 1	9.9 ± 0.5	4.5 ± 0.05	3.8 ± 1.3		
10		17 ± 3	11.0 ± 0.4	0.1 ± 0.07	0.1 ± 0.09	37% @ 100μM	N.D. @ 100μM

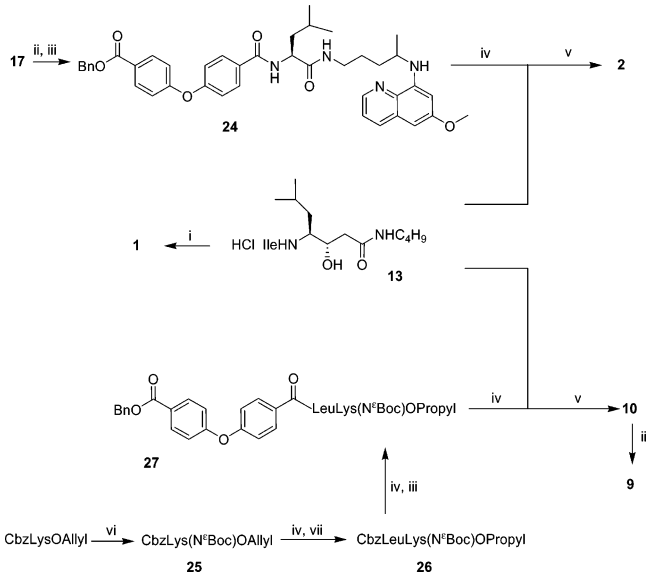
[a] Results are the mean ± SD. N.D., not detectable.

Scheme 1. Synthesis of Inhibitors **3**−**8**[a]



[a] Reagents: (i) LDA, EtOAc, $ZnBr_2$; (ii) NaOH; (iii) $C_4H_9NH_2$, HBTU, HOBt; (iv) HCl; (v) BocIleOH, HBTU, HOBt; (vi) BnBr, DMF; (vii) HBTU; (viii) BocLeuOH or BocAlaOH, HBTU, HOBt; (ix) CbzAlaOH or CbzLeuOH, HBTU, HOBt; (x) H_2, Pd−C; (xi) TFA; (xii) H_3PO_4.

Scheme 2. Synthesis of Inhibitors **1**, **2**, **9**, **10**[a]



[a] Reagents: (i) 2-(naphthalen-2-yloxy)acetic acid, HBTU; (ii) HCl; (iii) **14**, HBTU; (iv) H_2, Pd−C; (v) HBTU; (vi) Boc_2O; (vii) CbzLeuOH, HBTU, HOBt.

have a great deal of conformational freedom in the active site, and since it is exposed to the solvent, it is quite difficult to predict an active conformation. The model shows that the enzyme is easily able to accommodate large substituents after P3 and the 4,4′-oxybisbenzoic ring system is fitted nicely within the active site.

Correlation between Lipophilicity (log *P*) and Biological Activities. To verify whether lipophilicity and therefore cell penetration was correlated to the antiparasite activity, the degree of lipophilicity expressed as the log *P* value of PLM inhibitors was plotted against the ratio of the IC_{50} against the *P. falciparum* W2 strain in RBCs to the IC_{50} against PLM II (Figure 5). The

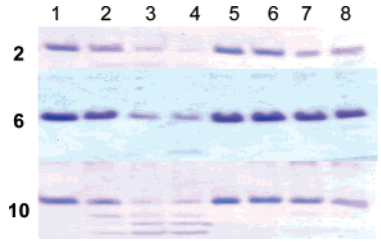


Figure 2. Hemoglobin degradation on SDS−PAGE, showing the effect of compounds **2**, **6**, and **10** on the cleavage of human hemeoglobin by PLM II: (lanes 1−4) hemoglobin incubated with the vehicle and PLM II for 0, 10, 30, and 60 min; (lanes 5−8) hemoglobin incubated with the inhibitor and PLM II for 0, 10, 30, and 60 min.

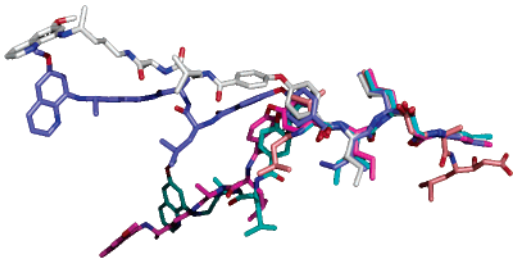


Figure 3. Superimposition of pepstatin A (pink) with some plausible conformations of compound **6** (various colors), showing the good superimposition of the statine moiety of the molecules and the conformational freedom of primaquine. The image was compiled using the PyMol molecular visualization system.[30]

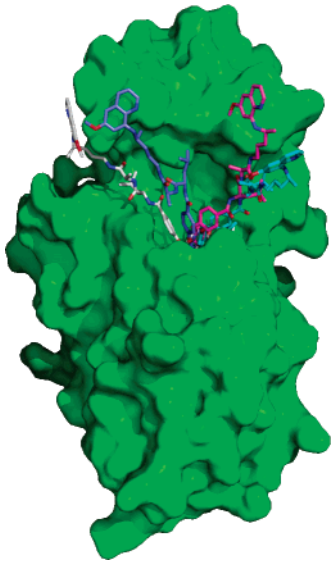


Figure 4. Surface plot of PLM II (green) in complex with some plausible conformations of compound **6** (various colors), showing the conformational freedom of primaquine. The image was compiled using the PyMol molecular visualization system.[30]

log *P* values were calculated using the Accelrys DS ViewerPro 5.0 property calculator (trial version) and are indicated as ALogP. The ratio of the IC_{50} values was chosen as an indicator of cell penetration of the synthesized inhibitors; the number will increase along with the impairment to cross cell membranes. A linear correlation ($r = 0.86$) between lipophilicity and activity ratio was obtained for all compounds but one (**9**).

Selectivity toward Human Cathepsin D and Cytotoxicity. For compounds **2**, **6**, and **10**, cathepsin D inhibition was negligible at the concentration required to inhibit PLM II by 50% (IC_{50}) (data not shown). At concentrations 100-fold higher, cathepsin D was inhibited by 30%, 50%, and 37%, respectively

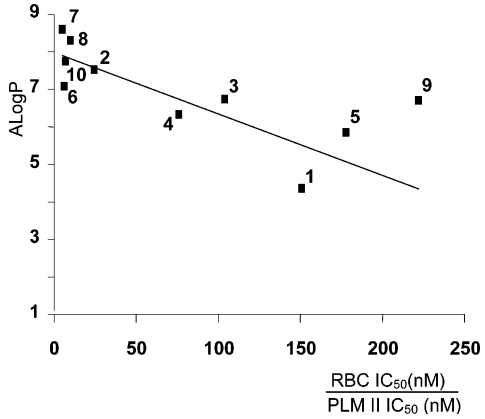


Figure 5. Correlation between lipophilicity (ALogP) and biological activity. A linear correlation ($r = 0.86$) between lipophilicity and activity ratio was obtained for all compounds but one (**9**). The log *P* values were calculated using Accelrys DS ViewerPro 5.0 property calculator (trial version) and are indicated as ALogP.

(Table 1), indicating a good selectivity for the plasmodial enzyme. Similarly, no antiproliferative activity was found when human skin fibroblasts were cultured in the presence of the compounds at concentrations as high as 100 μM. These results support for a negligible cytotoxic effect on mammalian cells at concentrations necessary to kill the parasite (below 1 μM).

Discussion

The PQ-statine compounds examined in this study represent the evolution of the first series of statine-based PLM II inhibitors designed by following the double-drug approach as devised by Romeo et al.[15] In agreement with previous results, it is confirmed that with this approach we have synthesized PLM II inhibitors that are much more active on *P. falciparum* than **1** (PS777621) and other transition state analogue inhibitors previously reported.[9−11]

Considering the redundancy of PLMs,[12,13] it is therefore necessary to design compounds able to inhibit two or more in this enzyme family in order to develop drugs active on *P. falciparum.* Some of the synthesized compounds, designed as PLM II inhibitors, are also strong inhibitors of PLM I, which is, in view of the recent literature, a prerequisite for developing new antimalarial agents.[13,14] In this regard, work is in progress to demonstrate the ability of PQ-statine inhibitors to block PLM IV and the orthologues from *P. malariae, P. ovale*, and *P. vivax* (manuscript in preparation).

By comparison of the PLM I inhibitory activity of the reference compound **1** with the effect exerted by compounds **2**−**10**, it is evident that most of them showed a remarkable improvement of activity. In particular the presence of the dipeptide influences the affinity for PLM I to a greater extent than PLM II with a preference for the Ala−Ala dipeptide (**5**), which shows an 80-fold improvement compared to compound **3**, which lacks the dipeptide linker.

It is noteworthy that most of the tested compounds possess an anti-PLM II activity much greater than compound **1**. With the exclusion of compounds **7** and **8**, any change to the peptide chain linking the PQ group to the 4,4′-oxybisbenzoic ring or the substitution of PQ with a propyl ester group did not bear heavy changes of PLM II K_i values (Table 1), indicating that PQ is not essential for PLM II inhibition. From modeling studies it looks like most of the conformations show solvent-exposed PQ and lack of interactions of the dipeptide linker with the enzyme. If this is true, no significant score differences will be seen because the score function mainly evaluates the interactions in the assigned boxes, i.e., in the active site cleft. Thus, structural modifications of this part of the molecule should not greatly affect the PLM II K_i values of the compounds. Regarding compounds **7** and **8**, which have the lowest affinity for PLM II, their interaction with the enzyme may be impaired by their high lipophilicity as deduced from the ALogP values (Figure 5).

The inhibition of *P. falciparum* growth in the erythrocytes was drastically improved by all compounds in comparison to **1**. IC_{50} values were below 1 μM for compounds **2**, **4**, **6**, and **10** with a noteworthy value of 100 nM for compound **10**, which represents the most active transition state analogue inhibitor to the best of our knowledge. Furthermore the PQ-statine compounds are equally active against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*, thus suggesting that the mechanism of action (i.e., PLM inhibition) and the mechanism for chloroquine resistance are independent.

The degree of PLM inhibition is not predictive of the ability to kill the parasite, since compounds with K_i values of 1−700 nM possess a range of potency against the parasite, falling within a much narrower interval (IC_{50} values of 0.1−4 μM). If parasite growth is not always correlated to PLM inhibition, then several factors could contribute to the antiplasmodial activity of these compounds and help explain the lack of correlation with inhibition of PLMs. They include (i) the ability to penetrate the cell and the food vacuole, where hemoglobin is degraded by PLMs I and II and (ii) the blockage of other proteolytic enzymes working in combination with PLM impairment.

In general, the crossing of cell membranes by a drug is facilitated by the hydrophobicity of the molecule. Figure 5 clearly shows that a linear correlation exists between log *P* values and the ratio of potencies against parasite and affinities against PLM II, thus indicating that PLMs are the main target of these compounds in the parasite. These results indicate that a good balance between inhibitory activity and lipophilicity is required in order to achieve efficacy in the cell.

The log *P* data indicate that for the compounds described here this rule is true for all but one (**9**). Compound **9** might undergo biotransformation, reducing its lipophilicity and thus interfering with cell bioavailability. We have previously shown that the breakdown of Lys-Xaa releases fragments much less potent than the parent compounds.[15]

The difference of the order of magnitude between enzymatic K_i and cell potency (IC_{50}) ranged between 10- and 400-fold. In light of the recent paper by Liu et al.,[14] it appears that bioavailability is not a limitation for killing cultured parasites, since pepstatin A was able to enter the food vacuole. Then it is possible that the potency of the compounds described is determined not only by their bioavailability but rather by the possibility that they inhibit not only plasmepsins but also other proteolytic enzymes (such as the falcipains).

The most promising inhibitors of *P. falciparum* growth (**2**, **6**, **10**) showed only negligible or no cytoxicity when tested on human fibroblasts cell line, indicating that these compounds interfere with mechanisms unique for the parasite; this observation was confirmed by the high selectivity of these compounds for PLMs in comparison to human cathepsin D.

The compounds under study, which have been designed on the basis of the double-drug approach, contain PQ, which is a drug active against liver-stage malaria parasites. Thus, compounds **2**−**8** containing PQ could also exert an inhibitory effect on this stage in addition to the intraerythrocytic effect. The in vivo studies should elucidate this hypothesis.

In conclusion, the evidence that some of the inhibitors reported possess very good efficacy against *P. falciparum* growth and a great selectivity in comparison to other inhibitors reported in the literature (0.1 vs 2−20 μM)[19−21] makes these novel compounds very promising for further development as antimalarial agents.

Experimental Section

Expression of Recombinant PLMs I and II. PLM I preparation will be described in full (Liu et al., manuscript in preparation). Briefly, expression of the recombinant proPfPLM I was induced at 37 °C in the BL21 Star (DE3) pLysS *E. coli* using 1 mM IPTG. After 3 h of induction, cell pellets were harvested and inclusion bodies were extracted and purified according to the procedure previously described.[22] Inclusion body materials were solubilized in 6 M urea, 50 mM sodium phosphate, pH 8.5, and 500 mM sodium chloride. Protein was refolded by dialysis against 20 mM Tris-HCl, pH 8.0, and further purified by anion exchange and size exclusion chromatography.

PLM II was prepared according to the procedure previously described,[23] with slight modifications. Briefly, pET-3a expression plasmid containing PLM II precursor gene was transformed into BL21-(DE3) pLysS *E. coli*. A single colony was used to inoculate 100 mL of LB medium. The culture was grown overnight at 30 °C, and an amount of 60 mL was used to inoculate 3 L of fresh LB. Cells were grown until the OD at 600 nm reached 0.4. They were then induced with IPTG at a final concentration of 1 mM. After a further 3 h of incubation, cells were harvested by centrifugation and resuspended in 100 mM Tris-HCl, pH 8, containing 0.2% Triton. After the addition of lysozyme (1 mg/mL), resuspended cells were incubated at 37 °C for 30 min. The volume was sonicated. Then solid urea was added to reach a final concentration of 500 mM and the solution was stirred at room temperature for 30 min. Inclusion bodies were harvested by centrifugation at 16000*g* for 10 min and then resuspended in the same buffer, stirred for 30 min, and spun as before. Inclusion bodies were solubilized in 6 M urea, 100 mM Tris-HCl, pH 8.5, and 32 mM β-mercaptoethanol. Protein was refolded by dialysis against 10 L of 10 mM Tris-HCl, pH 8.5. Protein concentration was determined according to the method of Bradford.[24] Protein was diluted to a final concentration of 0.5 mg/mL in 50% glycerol and stored at −20 °C.

Enzyme Inhibition Assays. PLM I Assay. For a tight binding (K_i = 50 pM to 10 nM) competitive inhibitor, the initial rates (AU/s) of enzymatic cleavage of a chromogenic peptide substrate (Lys-Pro-Ile-Leu-Phe-Nph-Arg-Leu) of known concentration (μM) in the presence of different concentrations (nM) of inhibitors were measured using a Cary 50 spectrophotometer (Varian).[22] The K_i value was determined by fitting the initial cleavage velocities and related inhibitor concentrations into the competitive tight binding inhibitor equation (J. F. Morrison, 1969):

$$v = \left\{\frac{0.5V_{max}/[E]}{\frac{K_m}{[S]}+1}\right\}\{[E]-[I]-K_i^{ap}+\sqrt{([E]-[I]-K_i^{ap})^2+4[E]K_i^{ap}}\}$$

where $K_i^{ap} = K_i([S]/K_m + 1)$, with the Enzfitter 1.05 program.

For a non-tight-binding (K_i = 50 nM to 10 μM) competitive inhibitor, the initial rates (AU/s) of enzymatic cleavage of at least six different concentrations of a chromogenic peptide substrate (Lys-Pro-Ile-Leu-Phe-Nph-Arg-Leu) in the presence of at least two different concentrations of inhibitors were measured and the K_i value was determined by fitting the initial hydrolysis rates and related substrate and inhibitor concentrations into the equation

$$v = \frac{[S]V_{max}}{[S]+K_m\left(1+\frac{[I]}{K_i}\right)}$$

of the single substrate−single inhibitor (competitive) program in the Enzyme Kinetic Module 1.0 of SigmaPlot 2000 (version 6.10) (SYSTAT Software Inc.).

PLM II Assay. Pro-PLM II was activated by addition of one-tenth volume of 100 mM sodium acetate buffer, pH 4.7, followed by incubation at 37 °C for 90 min. The enzyme activity of PLM II was evaluated spectrophotometrically at 300 nm by following the cleavage of the chromogenic substrate Lys-Glu-Phe-Val-Phe-NPhe-Ala-Leu-Lys (where NPhe is *p*-nitrophenylalanine), as described.[23]

Inhibitors were tested at 1−100 nM dissolved in DMSO (final concentration of 1% of the sample volume). IC_{50} values were obtained using Graph Pad Prism 4. The K_i values for PLM II were subsequently calculated by using

$$K_i = \frac{IC_{50}}{1+\frac{[S]}{K_m}}$$

and a K_m value determined according to Michaelis−Menten. Results are expressed as the mean ± standard deviation of at least two separate experiments performed in triplicate.

Cathepsin D Assay. Human liver cathepsin D was purchased from Sigma-Aldrich, Milan, Italy. The enzyme activity was measured as described for PLM II by incubating 0.714 U of protein with 27 μg of substrate in 100 mM sodium acetate buffer, pH 4.7. Final incubation volume was 0.6 mL. The compounds were tested against cathepsin D at concentrations 100-fold higher than that required to inhibit PLM II by 50% (IC_{50}).

Parasite Growth. The CQ-sensitive (D10) and the CQ-resistant (W2) strains of *P. falciparum* were sustained in vitro as described by Trager and Jensen.[25] Parasites were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (Gibco BRL, $NaHCO_3$ 24 mM) medium with the addition of 10% heat-inactivated A-positive human plasma, 20 mM Hepes (Euroclone), and 2 mM glutammine (Euroclone). All cultures were maintained at 37 °C in a standard gas mixture consisting of 1% O_2, 5% CO_2, 94% N_2.

Drug Susceptibility Assay on *P. falciparum*. Compounds were dissolved in either water (chloroquine) or DMSO and then diluted with a medium to achieve the required concentrations (final DMSO concentration of <1%, which is nontoxic to the parasite). Drugs were placed in 96-well flat-bottom microplates (COSTAR), and serial dilutions were made. Asynchronous cultures with parasitemia of 1−1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 72 h at 37 °C. Parasite growth was determined spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (LDH) according to a modified version of Makler's method in control and drug-treated cultures.[26] Antiplasmodial activity is expressed as the 50% inhibitory concentrations (IC_{50}). Each IC_{50} value is the mean ± standard deviation of at least three separate experiments performed in duplicate.

Hemoglobin Degradation Assay by PLM II in the Presence of PQ-Statine Compounds. The ability of the most active compounds to prevent the hemoglobin degradation by PLM II was assayed by SDS−PAGE.[27] Briefly, hemoglobin (10 μg) was incubated with preactivated PLM II (200 ng) at 37 °C, pH 4.7, in a final digest volume of 16 μL of 100 mM sodium acetate buffer and in the presence of the vehicle or the PLM II inhibitor for 10, 30, and 60 min. The reaction was stopped by the addition of SDS loading dye. Samples were denatured by boiling for 5 min and loaded onto 18% polyacrylamide gel.

Cytotoxicity Assay. Cytotoxicity was evaluated in human fibroblasts from skin biopsies. Fibroblasts (8×10^4 per well) were grown in 24-well plates with DMEM containing 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine as previously described.[28] Cell proliferation was followed by the MTT (3-

[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test[29] in the absence or in the presence of the tested compounds (up to 100 μM).

Modeling of PLM II with PQ-Statine Inhibitors. The 3D structural models of compound **6**, one of the most promising compounds (Table 1), were constructed from pepstatin A using the X-ray crystal coordinates of pepstatin A in complex with plasmepsin II (PDB entry 1XDH) as a starting point. The Val residue in P2 of pepstatin A was changed in Ile and substituted with 4,4′-oxybisbenzoic acid. The C-terminal residues AlaSta of pepstatin A were removed, and the *n*-butylamine chain was added. This molecule was minimized within the active site allowing all atoms to move, while the enzyme was kept fixed. The LeuAlaPQ part of the molecule was separately built and minimized in an extended conformation. The two parts of **6** were then joined and minimized in the active site as previously described. Conformers of **6** were manually generated by rotating the bonds of the 4,4′-oxybisbenzoic ring system while leaving the LeuAlaPQ part of the molecule fixed. Each conformer was minimized within the PLM II allowing all atoms of **6** to move, while the enzyme was kept fixed. The minimizations were performed in Accelrys DS ViewerPro 5.0 Conformer (trial version) with the steepest descent. All minimizations converged before 10 000 iterations.

General Methods. NMR spectra were recorded on a Varian 300 MHz instrument using $CDCl_3$, CD_3OD, or DMSO-d_6 as solvents. TMS was used as reference. Peaks were assigned with 2D COSY experiments and are consistent with the proposed structures. Melting points were determined with a Büchi apparatus and are uncorrected. TLC was carried out on Merck precoated 60 F254 plates using UV light and dipping with ethanol/phosphomolybdic acid (10%) or a solution of 0.5% ninhydrin in ethanol for visualization. Flash column chromatography was performed using silica gel 60 (0.040−0.063 mm, Merck). Organic phases were dried over anhydrous sodium sulfate. Concentrations were performed under diminished pressure (1−2 kPa) at a bath temperature of 40 °C. High-resolution mass spectra (ESI) were recorded on a Fourier transform ion cyclotron resonance mass spectrometer APEX II and Xmass software (Bruker Daltonics). Mass spectra (ESI, positive ions) were recorded on a LCG Advantage (Thermo Finnigan).

General Synthetic Procedures. Procedure A. Coupling. The carboxylic acid (1.1 equiv) and the amine (1.0 equiv) are dissolved under nitrogen in an appropriate solvent (DMF, dichloromethane, or acetonitrile) (5 mL/mmol). *N*-Methylmorpholine was then added followed by hydroxybenzotriazole solution (HOBt) (1.5 equiv). The resulting solution was cooled at 0 °C, and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) was added (1.1 equiv). The mixture was then stirred at room temperature overnight. Dichloromethane was then added, and the organic phase was washed with citric acid (5%), saturated $NaHCO_3$, water, and brine, dried, filtered, and concentrated.

General Synthetic Procedures. Procedure B. Removal of the Boc Group (HCl). After cooling the Boc-protected compound at 0 °C, 4 N HCl in dioxane was added (1 mL/100 mg of compound). The mixture was stirred at room temperature for 30 min. After this time the mixture was concentrated and the residue washed three times with ethyl ether and used in the next step without any further purification or characterization.

General Synthetic Procedures. Procedure C. Removal of the Boc Group (TFA). To the Boc-protected compound was added dropwise at room temperature a 1:1 solution of TFA/dichloromethane (4 mL/mmol). After being stirred for 40−50 min, the mixture was concentrated and washed three times with ethyl ether and filtered, and the solid obtained was used in the next step without any further purification or characterization.

General Synthetic Procedures. Procedure D. Removal of the Cbz Group by Catalytic Hydrogenation. The Cbz-protected compound was dissolved in methanol (10 mL/mmol), and then Pd/C (80 mg/mmol) added. When the reaction was completed (30−40 min), the mixture was filtered and washed with methanol and the residue was concentrated and used in the next step without any further purification or characterization.

(3*S*,4*S*)-4-(*tert*-Butoxycarbonylamino)-3-hydroxy-6-methylheptanoic Acid Ethyl Ester (BocStaOEt) (11). Anhydrous THF (24 mL) and LDA (2 N, 28.3 mL, 56.6 mmol) were cooled under nitrogen atmosphere at −80 °C. Ethyl acetate (5.6 mL, 56.6 mmol) was then added followed by a 0 °C solution of anhydrous $ZnBr_2$ (12.7 g, 56.6 mmol) in anhydrous THF (24 mL). A −78 °C solution of Boc-leucinal[17] (1.81 g, 8.4 mmol) in anhydrous THF (6 mL) was then added, and the mixture was stirred at −78 °C for 10 min and finally allowed to reach room temperature overnight. After the reaction mixture was cooled at 0 °C, 25 mL of a solution of aqueous saturated NH_4Cl and acetic acid (9:1) was added and extracted with cooled ethyl ether (four times). The organic phase was washed with 1 N HCl (2 times) and with brine, dried over sodium sulfate, filtered, and concentrated under vacuum. The brownish oil was purified by silica gel chromatography (cyclohexane/ethyl acetate (90:10, 85:15, 70:30) to give **11** as a white solid (253 mg, 0.84 mmol, 10%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.82−1.02 (9H, m), 1.02−1.74 (12H, m), 2.50 (2H, m), 3.60 (1H, bm), 4.00 (1H, bm), 4.15 (2H, q), 4.73 (1H, bd). Anal. ($C_{15}H_{29}NO_5$) C, H, N.

(2*S*,3*S*)-1-((3*S*,4*S*)-1-(Butylamino)-3-hydroxy-6-methyl-1-oxoheptan-4-ylamino)-3-methyl-1-oxopentan-2-ylcarbamic Acid *tert*-Butyl Ester (BocIleStaNHC$_4$H$_9$) (12). A solution of 2 N NaOH (0.84 mmol, 0.42 mL) was added dropwise to a solution of Boc-StaOEt (253 mg, 0.84 mmol) in dioxane/water (3:1, 6 mL) cooled to 0 °C. The mixture was stirred at room temperature for 1.5 h. After completion, the reaction mixture was cooled to 0 °C and cooled 2 N HCl was added until acidic pH was attained. Dioxane was removed by evaporation, and the aqueous phase was extracted with dichloromethane (three times). The organic phases were dried and concentrated, and the product was reacted with butylamine (61.4 mg, 0.84 mmol) under general procedure A. The reaction mixture was extracted with ethyl acetate, dried, and concentrated to give a crude product (195 mg), which was used without any further purification in the next reaction.

The crude product was reacted under general procedure B, followed by reaction under general procedure A with BocIleOH (194 mg, 0.84 mmol) to give after crystallization with ethyl acetate product **12** (257 mg, 0.58 mmol, 32%). R_f = 0.4 (cycloexane/ethyl acetate, 1:1). Mp 174−175 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.80−1.04 (15H, m), 1.26−1.40 (4H, m), 1.43 (9H, s), 1.41−1.68 (5H, m), 1.91 (1H, m), 2.20 (1H, d, J = 3.67 Hz), 2.25 (1H, d, J = 3.73 Hz), 3.17−2.29 (2H, m), 3.91 (2H, m), 3.98 (1H, m), 4.90 (1H, d, J = 7.12 Hz), 6.36 (1H, bm), 6.41 (1H, d J = 9.65 Hz). Anal. ($C_{23}H_{45}N_3O_5$) C, H, N.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-Amino-3-methylpentanamido)-*N*-butyl-3-hydroxy-6-methylheptanamide (IleStaNHC$_4$H$_9$ HCl) (13). Product **12** (150 mg, 0.34 mmol) was reacted under general procedure B to give **13** as a white solid purified by digestion with ethyl ether. The product was used in the next reaction without any further purification or characterization.

4-(4-(Benzyloxycarbonyl)phenoxy)benzoic Acid (14). 4,4′-Oxybisbenzoic acid (2.00 g, 7.74 mmol) was dissolved at room temperature in DMF (20 mL), and TEA was added (1.02 g, 10.1 mmol). Benzyl bromide (1.14 g, 10.1 mmol) was then added dropwise, and the mixture was heated at 60 °C under stirring for 3 h. The mixture was then cooled at room temperature and extracted with ethyl acetate (3 times). The organic phase washed with water and brine, dried, and concentrated.

Product **14** was obtained as a white solid (631 mg, 2.44 mmol, 32%) after purification of the crude reaction mixture by silica gel flash chromatography (CH_2Cl_2/MeOH, 95:5) followed by digestion with ethyl ether. R_f = 0.45 (CH_2Cl_2/CH_3OH, 90:10). ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5, 300 MHz) δ: 5.31 (2H, s), 7.02 (4H, m), 7.38 (5H, m), 8.02 (4H, m).

4-(4-(((2*S*,3*S*)-1-((3*S*,4*S*)-1-(Butylamino)-3-hydroxy-6-methyl-1-oxoheptan-4-ylamino)-3-methyl-1-oxopentan-2-yl)carbamoyl)-phenoxy)benzoic Acid Benzyl Ester (15). By reaction under general procedure A of amine **13** (131 mg, 0.49 mmol) and acid **14** (172 mg, 0.49 mmol), product **15** was obtained (325 mg, 0.48 mmol) as a white solid and used in the next reaction without purification. R_f = 0.5 (CH_2Cl_2/CH_3OH, 95:5). Mp 173 °C. ^{1}H NMR

($CDCl_3$, 300 MHz) δ: 0.81−1.00 (12H, m), 1.03 (3H, d, J = 6.66 Hz), 1.12−1.74 (9H, m), 2.03 (1H, m), 2.29 (1H, dd, J = 14.9, 4.0 Hz), 2.40 (1H, dd, J = 14.7, 9.1 Hz), 3.24 (2H, m), 3.92 (1H, m), 4.02 (1H, m), 4.48 (1H, t), 5.36 (2H, s), 6.45 (1H, m), 6.52 (1H, d, J = 9.3 Hz), 6.78 (1H, d, J = 8.2 Hz), 6.95−7.14 (4H, m), 7.32−7.54 (5H, m), 7.80 (2H, d), 8.08 (2H, d, J = 8.6 Hz). MS (M + Na)$^+$: 696.5.

***tert*-Butyl (*S*)-1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-1-oxopropan-2-ylcarbamate (4′-*N*-(Boc-alanyl)primaquine) (16).** Compound **16** was prepared according to general procedure A, by reacting primaquine diphosphate (179 mg, 0.5 mmol) with Boc-alanine (95 mg, 0.5 mmol). The product was obtained (171 mg, 0.4 mmol, 79%) as a brownish amorphous solid and used in the next reaction without further purification. R_f = 0.5 (CH_2Cl_2/CH_3-OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.06−2.25 (19H, m), 3.27 (2H, bm), 3.62 (1H, m), 3.95 (3H, s), 4.27 (1H, bs), 5.45 (1H, bm), 6.55 (2H, m), 7.26 (3 H, m), 7.69 (1H, bd), 8.57 (1H, bd). MS (M + Na)$^+$: 453.2

(*S*)-1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-4-methyl-1-oxopentan-2-ylcarbamic Acid *tert*-Butyl Ester (4′-*N*-(Boc-leucyl)primaquine) (17). Compound **17** was prepared according to general procedure A by reacting primaquine diphosphate (300 mg, 0.84 mmol) with Boc-leucine (209 mg, 0.84 mmol). The product was obtained (330 mg, 0.698 mmol, 83%) as a yellow amorphous solid and used in the next reaction without further purification. R_f = 0.55 (CH_2Cl_2/CH_3OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.92 (6H, m), 1.25 (3H, m), 1.41 (9H, m), 1.54−1.82 (7H, bm), 3.27 (2H, bm), 3.61 (1H, bm), 3.89 (3H, s), 4.08 (1H, bm), 4.99 (1H, bs), 6.32 (2H, d), 7.34 (1H, bs), 7.97 (1H, bs), 8.53 (1H, d). MS (M + Na)$^+$: 495.4.

((*S*)-1-{(*S*)-1-[4-(6-Methoxyquinolin-8-ylamino)pentylcarbamoyl]ethylcarbamoyl}ethyl)carbamic Acid Benzyl Ester (4′-*N*-(Cbz-alanylalanyl)primaquine) (18). Compound **18** was obtained by reacting compound **16** (93 mg, 0.217 mmol) according to general procedure B, followed by general procedure A with Cbz-alanine (48 mg, 0.217 mmol). The product was obtained (86 mg, 0.161 mmol, 74%) as a green amorphous solid and purified by digestion with ether. R_f = 0.3 (CH_2Cl_2/CH_3OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.02−1.42 (7H, m), 1.44−1.74 (6H, bs), 3.21 (2H, bm), 3.56 (1H, bm), 3.83 (3H, s), 4.11 (1H, bm), 4.35 (1H, bm), 5.01 (2H, s), 5.25 (1H, bs), 5.98 (1H, bs), 6.26 (2H, d), 6.60 (1H, bs), 7.26 (7H, m), 7.88 (1H, bd), 8.46 (1H, m). MS (M + Na)$^+$: 558.2.

((*S*)-1-{(*S*)-1-[4-(6-Methoxyquinolin-8-ylamino)pentylcarbamoyl]ethylcarbamoyl}-3-methylbutyl)carbamic Acid Benzyl Ester (4′-*N*-(Cbz-leucylalanyl)primaquine) (19). Compound **19** was obtained by reacting compound **16** (56 mg, 0.13 mmol) according to general procedure B followed by general procedure A with Cbz-leucine (35 mg, 0.13 mmol). The product was obtained (37 mg, 0.066 mmol, 51%) as a green amorphous solid after purification by silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 98:2). R_f = 0.4 (CH_2Cl_2/CH_3OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.84 (6H, m), 1.13−1.35 (6H, m), 1.48−1.80 (7H, bm), 3.20 (2H, bm), 3.56 (1H, bm), 3.83 (3H, s), 4.08 (1H, bm), 4.34 (1H, bm), 5.01 (2H, s), 5.14 (1H, bs), 5.96 (1H, bs), 6.25 (2H, d), 6.58 (1H, bs), 7.26 (7H, m), 7.87 (1H, bd), 8.46 (1H, m). MS (M + Na)$^+$: 600.4.

(*S*)-1-((*S*)-1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-4-methyl-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2-ylcarbamic Acid Benzyl Ester (4′-*N*-(Cbz-leucylleucyl)primaquine) (20). Compound **20** was obtained by reacting compound **17** (130 mg, 0.275 mmol) according to general procedure B followed by general procedure A with Cbz-leucine (73 mg, 0.275 mmol). The product was obtained (130 mg, 0.21 mmol, 76%) as a yellow amorphous solid after purification by silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 98:2). R_f = 0.4 (CH_2Cl_2/CH_3-OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.90 (12H, s), 1.28 (3H, m), 1.39−1.82 (10H, bm), 3.23 (2H, bm), 3.63 (1H, bd), 3.90 (3H, s), 4.18 (1H, bm), 4.45 (1H, bm), 5.06 (2H, m), 6.24−6.61 (2H, bm), 7.30 (7H, m), 8.53 (1H, s). MS (M + Na)$^+$: 624.2

{(*S*)-5-((*S*)-2-Benzyloxycarbonylaminopropionylamino)-5-[4-(6-methoxyquinolin-8-ylamino)pentylcarbamoyl]pentyl}carbamic Acid *tert*-Butyl Ester (4′-*N*-[Cbz-alanyl(*N*$^\epsilon$-Boc-lysyl)]primaquine) (21). Compound **21** was prepared according to general procedure A by reacting 4′-*N*-(*N*$^\epsilon$-Boc-lysyl)primaquine[6] (149 mg, 0.306 mmol) with Cbz-alanine (68 mg, 0.306 mmol). The product was obtained (113 mg, 0.16 mmol, 46%) as a yellow amorphous solid after purification by silica gel gradient chromatography (CH_2-Cl_2/CH_3OH, 99:1 to 98:2). R_f = 0.4 (CH_2Cl_2/CH_3OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.83−2.26 (25H, m), 2.89−3.30 (3H, bm), 3.33−3.75 (2H, bm), 3.95 (3H, s), 4.32 (1H, bm), 4.59 (1H, bm), 4.85 (1H, bm), 5.08 (2H, s), 6.57 (2H, s), 7.32 (8H, m), 7.69 (1H, m), 8.53 (2H, m). MS (M + Na)$^+$: 715.5.

{(*S*)-5-((*S*)-2-Benzyloxycarbonylamino-4-methylpentanoylamino)-5-[4-(6-methoxyquinolin-8-ylamino)pentylcarbamoyl]pentyl}carbamic Acid *tert*-Butyl Ester (4′-*N*-[Cbz-leucyl(*N*$^\epsilon$-Boc-lysyl)]primaquine) (22). Compound **22** was prepared according to general procedure A by reacting 4′-*N*-(*N*$^\epsilon$-Boc-lysyl)PQ[6] (97 mg, 0.2 mmol) with Cbz-leucine (53 mg, 0.2 mmol). The product was obtained (102 mg, 0.14 mmol, 69%) as a yellow amorphous solid after purification by silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 98:2). R_f = 0.5 (CH_2Cl_2/CH_3OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.88 (6H, m), 1.00−1.78 (25 H, m), 2.89−3.30 (3H, bm), 3.33−3.75 (2H, bm), 3.95 (3H, s), 4.33 (1H, bm), 4.57 (1H, bm), 4.82 (1H, bm), 5.08 (2H, s), 6.57 (2H, s), 7.32 (9H, m), 8.53 (2H, m). MS (M + Na)$^+$: 757.6.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-((*S*)-1-(6-(*tert*-Butoxycarbamoyl)-1-(4-(6-methoxyquinolin-8-ylamino)pentylamino)-1-oxohexan-2-ylamino)-1-oxopropan-2-yl)carbamoyl)phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (23). Compound **23** was obtained by reacting a mixture of 4′-*N*-[Cbz-alanyl(*N*$^\epsilon$-Boc-lysyl)]PQ (**21**) (69 mg, 0.1 mmol) and benzyl ester **15** (67 mg, 0.1 mmol) according to general procedure D followed by general procedure A. The product was obtained (44 mg, 0.039 mmol, 39%) as a yellow solid after crystallization from ethyl acetate. R_f = 0.5 (CH_2Cl_2/CH_3OH, 90:10). Mp 190 °C. ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5, 300 MHz) δ: 0.65−1.04 (15H, m), 1.06−1.82 (34H, m), 1.91 (1H, bm), 2.20 (2H, d), 2.90 (2H, bm), 3.07−3.26 (4H, m), 3.57 (1H, bm), 3.84 (1H, s), 3.74−3.89 (2H, m), 4.22−4.29 (1H, bm), 4.36 (1H, t), 4.51−4.61 (1H, bm), 6.23−6.35 (2H, d), 6.99 (4H, m), 7.79 (5H, t), 8.41−8.51 (2H, bm). MS (M + Na)$^+$: 1146.5.

(3*S*,4*S*)-*N*-Butyl-3-hydroxy-6-methyl-4-((2*S*,3*S*)-3-methyl-2-(2-(naphthalen-2-yloxy)acetamido)pentanamido)heptanamide (1). Product **1** was obtained by reacting amine **13** (34.7 mg, 0.25 mmol) with 2-(naphthalen-2-yloxy)acetic acid (50 mg, 0.25 mmol) according to general procedure A and purified by crystallization from ethyl acetate to give a white solid (61 mg, 0.12 mmol, 47%). R_f = 0.5 (CH_2Cl_2/CH_3OH, 95:5). Mp 179 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.79−1.19 (15H, m), 1.22−1.83 (9H, m), 1.97 (1H, bm), 2.26 (2H, m), 3.24 (2H, m), 3.88−4.05 (2H, bm), 4.33 (1H, m), 4.65 (2H, s), 6.39 (1H, bd), 7.26 (3H, m), 7.47 (2H, m), 7.78 (2H, m). HRMS calcd for $C_{30}H_{45}N_3O_5Na$ (M + Na)$^+$: 550.3251. Found: 550.3242.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((4-(6-Methoxyquinolin-8-ylamino)-pentyl)carbamoyl)phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (3). Compound **3** was obtained by reacting benzyl ester **15** (128 mg, 0.19 mmol) according to general procedure D followed by general procedure A with primaquine diphosphate (74 mg, 0.16 mmol). The product was obtained (92 mg, 0.11 mmol, 69%) as a yellow solid after silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 95:5) followed by digestion in ether. R_f = 0.38 (CH_2Cl_2/CH_3OH, 95:5). Mp 167 °C (dec). ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 0.73−1.01 (15H, m), 1.01−1.43 (10H, m), 1.43−1.77 (6H, bm), 1.92 (1H, bm), 2.07 (2H, m), 3.00 (2H, m), 3.28 (2H, m), 3.63 (1H, m), 3.76−3.89 (5H, m), 4.28 (1H, t), 4.90 (1H, d), 6.14 (1H, d), 6.26 (1H, m), 6.46 (1H, m), 7.04−7.13 (4H, m), 7.41 (1H, q), 7.49 (1H, d), 7.68 (1H, t), 7.81−7.97 (4H, m), 8.06 (1H, dd), 8.33 (1H, d), 8.46 (1H, t), 8.51 (1H, m). HRMS calcd for $C_{47}H_{64}N_6O_7Na$ (M + Na)$^+$: 847.4729. Found: 847.4706.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-((*S*)-1-(6-Amino-1-(4-(6-methoxyquinolin-8-ylamino)pentylamino)-1-oxohexan-2-ylamino)-1-

oxopropan-2-yl)carbamoyl)phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (4). Compound **23** (30 mg, 0.027 mmol) was reacted under general procedure C. The resulting salt was dissolved in methanol, and H_3PO_4 (0.054 mmol) was added. The mixture was dried, and product **4** (30 mg, 0.025 mmol, 91%) was obtained as an orange solid after crystallization from methanol/ether. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 0.70−1.01 (15H, m), 1.97−1.76 (25H, m), 1.92 (1H, bm), 2.08 (2H, m), 2.72 (2H, s), 2.91−3.16 (4H, m), 3.60 (1H, bm), 3.75−3.90 (5H, m), 4.17 (1H, m), 4.29 (1H, t), 4.41 (1H, t), 6.08 (1H, d), 6.24 (1H, m), 6.45 (1H, d), 7.09 (5H, d), 7.43 (2H, m), 7.67 (1H, t), 7.94 (7H, m), 8.33 (1H, m), 8.51 (1H, m). HRMS calcd for $C_{56}H_{81}N_9O_9Na$ $(M + Na)^+$: 1046.6050. Found: 1046.6059.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-((*S*)-1-(1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-1-oxopropan-2-ylamino)-1-oxopropan-2-yl)carbamoyl)phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (5). Compound **5** was obtained by reacting a mixture of 4′-*N*-(Cbz-alanylalanyl)-PQ (**18**) (35 mg, 0.065 mmol) and benzyl ester **15** (44 mg, 0.065 mmol) according to general procedure D followed by general procedure A. The product was obtained (32 mg, 0.033 mmol, 50%) as a yellow solid after crystallization from ethyl acetate. $R_f = 0.65$ (CH_2Cl_2/CH_3OH, 90:10). Mp 194 °C. ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5, 300 MHz) δ: 0.63−1.00 (15H, m), 1.03−2.04 (23H, m), 2.24 (2H, m), 3.16−3.23 (4H, m), 3.52 (1H, m), 3.88 (3H, s), 3.77−3.95 (2H, m), 4.38 (1H, bd), 4.50 (1H, m), 4.65 (1H, m), 6.51 (2H, s), 6.89 (4H, m), 7.75 (5H, m), 8.49 (1H, d), 8.60 (1H, dd). HRMS calcd for $C_{53}H_{74}N_8O_9Na$ $(M + Na)^+$: 989.5471. Found: 989.5451.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-((*S*)-1-(1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-1-oxopropan-2-ylamino)-4-methyl-1-oxopentan-2-yl)carbamoyl)phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (6). Compound **6** was obtained by reacting a mixture of 4′-*N*-(Cbz-leucylalanyl)PQ (**19**) (29 mg, 0.052 mmol) and benzyl ester **15** (35 mg, 0.052 mmol) according to general procedure D followed by general procedure A. The product was obtained (20 mg, 0.02 mmol, 39%) as a yellow solid after crystallization from ethyl acetate. $R_f = 0.5$ (CH_2Cl_2/CH_3OH, 90:10). Mp 204 °C. ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5, 300 MHz) δ: 0.67−1.05 (21H, m), 1.07−1.76 (22H, m), 1.88 (1H, bm), 2.20 (2H, d), 3.06−3.26 (4H, m), 3.55 (1H, bm), 3.84 (3H, s), 3.72−3.95 (2H, m), 4.22−4.41 (2H, m), 4.59 (1H, bm), 6.24 (2H, s), 7.00 (4H, m), 7.79 (5H, m), 8.44 (2H, bd). HRMS calcd for $C_{56}H_{80}N_8O_9Na$ $(M + Na)^+$: 1031.5940. Found: 1031.5923.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-((*S*)-1-(6-(*tert*-Butoxycarbamoyl)-1-(4-(6-methoxyquinolin-8-ylamino)pentylamino)-1-oxohexan-2-ylamino)-4-methyl-1-oxopentan-2-yl)carbamoyl)phenoxy)-benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (7). Compound **7** was obtained by reacting a mixture of 4′-*N*-[Cbz-leucyl(N^ϵ-Boc-lysyl)]primaquine **22** (41 mg, 0.056 mmol) and benzyl ester **15** (38 mg, 0.056 mmol) according to general procedure D followed by general procedure A. The product was obtained (17 mg, 0.015 mmol, 26%) as a yellow solid after silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 96:4) followed by digestion in ethyl ether. $R_f = 0.4$ (CH_2Cl_2/CH_3OH, 90:10). Mp 203 °C. ^{1}H NMR ($CDCl_3$/CD_3OD 95:5, 300 MHz) δ: 0.72−1.03 (21H, m), 1.06−1.96 (35H, m), 2.20 (2H, d), 3.03−3.29 (6H, m), 3.56 (1H, m), 3.89 (3H, s), 3.80−3.93 (2H, m), 4.35−4.37 (2H, m), 4.63 (1H, m), 6.52 (2H, m), 6.99 (4H, m), 7.75−7.91 (5H, m), 8.40−8.44 (1H, bs), 8.57 (1H, m). HRMS calcd for $C_{64}H_{95}N_9O_{11}Na$ $(M + Na)^+$: 1188.7043. Found: 1188.7026.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-((*S*)-1-(1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-4-methyl-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2*S*-yl)carbamoyl)phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (8). Compound **8** was obtained by reacting a mixture of 4′-*N*-(Cbz-leucylleucyl)primaquine **20** (47 mg, 0.076 mmol) and benzyl ester **15** (51 mg, 0.076 mmol) according to general procedure D followed by general procedure A. The product was obtained (14 mg, 0.013 mmol, 17%) as a yellow solid after silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 96:4) followed by digestion in ether. $R_f = 0.5$ (CH_2Cl_2/CH_3OH, 90:10). Mp 211 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.72−1.06 (27H, m), 1.09−1.99 (22H, m), 1.91 (1H, bs), 2.21 (2H, m), 3.05−3.43 (4H, m), 3.66 (1H, bd), 3.78−3.95 (2H, m), 3.91 (3H, s), 4.34−4.53 (2H, m), 4.67 (1H, bt), 6.53 (2H, s), 6.98 (4H, m), 7.80 (5H, m), 8.57 (2H, m). HRMS calcd for $C_{59}H_{86}N_8O_9Na$ $(M + Na)^+$: 1073.6410. Found: 1073.6400.

4-(4-(((*S*)-1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-4-methyl-1-oxopentan-2-yl)carbamoyl)phenoxy)benzoic Acid Benzyl Ester (24). Compound **24** was obtained by reacting compound **17** (125 mg, 0.264 mmol) according to general procedure B followed by general procedure A with acid **14** (92 mg, 0.264 mmol). The product was obtained (142 mg, 0.2 mmol, 78%) as a yellow amorphous solid after purification by silica gel chromatography (CH_2Cl_2/CH_3OH, 98:2). $R_f = 0.5$ (CH_2Cl_2/CH_3OH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.95 (6H, m), 1.26 (3H, m), 1.55−1.80 (7H, bm), 3.23 (2H, bm), 3.60 (1H, bm), 3.88 (3H, s), 4.63 (1H, bs), 5.36 (2H, s), 5.98 (1H, bs), 6.27 (1H, bd), 6.72 (1H, bs), 7.00 (5H, m), 7.43 (7H, m), 7.78 (3H, m), 8.05 (2H,m), 8.51 (1H, m). MS $(M + Na)^+$: 725.1.

(3*S*,4*S*)-4-((2*S*,3*S*)-2-(4-(4-((*S*)-(1-(4-(6-Methoxyquinolin-8-ylamino)pentylamino)-4-methyl-1-oxopentan-2-yl)carbamoyl)-phenoxy)benzamido)-3-methylpentanamido)-3-hydroxy-6-methylheptanoic Acid Butylamide (2). Compound **2** was obtained by reacting benzyl ester **24** (62 mg, 0.09 mmol) according to general procedure D followed by general procedure A with amine **13** (24.0 mg, 0.09 mmol). The product was obtained (24 mg, 0.025 mmol, 28%) as a yellow solid after silica gel gradient chromatography (CH_2Cl_2/CH_3OH, 99:1 to 95:5) followed by digestion in ethyl ether. $R_f = 0.6$ (CH_2Cl_2/CH_3OH, 95:5). Mp 193 °C. ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5, 300 MHz) δ: 0.73−1.00 (21H, m), 1.08−1.96 (20H, m), 2.18 (2H, bd), 3.12 (2H, m), 3.60 (2H, bm), 3.87 (3H, s), 3.76−3.90 (3H, m), 4.35 (1H, m), 4.66 (1H, bm), 6.50 (2H, bs), 6.98 (4H, m), 7.74 (5H, m), 8.45−8.59 (2H, bm). HRMS calcd for $C_{53}H_{75}N_7O_8Na$ $(M + Na)^+$: 960.5569. Found: 960.5558.

(*S*)-2-Benzyloxycarbonylamino-6-*tert*-butoxycarbonylamino-hexanoic Acid Allyl Ester (Cbz-(N^ϵ-Boc-lysine allyl ester) (25). Cbz-lysine allyl ester[18] (1.25 g, 3.50 mmol) was dissolved in dichloromethane (25 mL). TEA (0.53 mL, 3.50 mmol) was then added dropwise under stirring, followed by di-*tert*-butyl dicarbonate (764 mg, 3.50 mmol). After being stirred for 12 h at room temperature, the reaction mixture was washed with HCl (1 M, ×3), $NaHCO_3$ (saturated, ×3), H_2O (×3), and brine, dried, and concentrated to give **24**, which was used in the next reaction without purification (1.50 g, 2.60 mmol). $R_f = 0.8$ (CH_2Cl_2/MeOH, 95: 5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.42 (9H, s), 1.48 (2H, bm), 1.60−1.76 (2H, bm), 1.77−1.93 (2H, bm), 3.10 (2H, t), 4.37 (1H, bt), 4.64 (2H, m), 5.10 (2H, s), 5.29 (2H, m), 5.35 (1H, s), 5.87 (1H, bm), 7.53 (5H, m). MS $(M + Na)^+$: 443.3.

(*S*)-2-((*S*)-2-Benzyloxycarbonylamino-4-methylpentanoylamino)-6-*tert*-butoxycarbonylaminohexanoic Acid Propyl Ester (Cbz-leucyl-N^ϵ-Boc-lysine Propyl Ester) (26). Compound **26** was obtained by reacting compound **25** (500 mg, 1.20 mmol) according to general procedure D followed by general procedure A with Cbz-leucine (1.18 mmol, 313 mg). The product was obtained (500 mg, 1.15 mmol) as a white solid and was used in the next reaction without purification. $R_f = 0.6$ (CH_2Cl_2/MeOH, 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.94 (9H, m), 1.21−1.36 (2H, bm), 1.43 (9H, s), 1.49−1.90 (9H, bm), 3.05 (2H, bt), 4.09 (2H, t), 4.20 (1H, bt), 4.57 (1H, bt), 5.11 (2H, s), 7.34 (5H, m). MS $(M + Na)^+$: 558.6.

4-{4-[(*S*)-1-((*S*)-5-*tert*-Butoxycarbonylamino-1-propoxycarbonylpentylcarbamoyl)-3-methylbutylcarbamoyl]phenoxy}-benzoic Acid Benzyl Ester (27). Product **27** was obtained from **26** (500 mg, 1.15 mmol) according to general procedure D followed by general procedure A with acid **14** and purified by silica gel flash chromatography (cyclohexane/ethyl acetate, 7:3) (380 mg, 0.52 mmol, 45%). $R_f = 0.5$ (CH_2Cl_2/MeOH, 97: 3). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.93 (7H, m), 1.35−1.52 (13H, bm), 1.63−1.88 (9H, bm), 3.02 (2H, t), 4.11 (2H, t), 4.58 (1H, bt), 4.70 (1H, bt), 5.36

(2H, s), 7.06 (4H, m), 7.40 (5H, m), 7.84 (2H, m), 8.07 (2H, m). MS $(M + Na)^+$: 754.2.

(*S*)-2-(2*S*-(4-(4-(((2*S*,3*S*)-1-((3*S*,4*S*)-1-(Butylamino)-3-hydroxy-6-methyl-1-oxoheptan-4-ylamino)-3-methyl-1-oxopentan-2-yl)-carbamoyl)phenoxy)benzamido)propanamido)-6-(*tert*-butoxy-carbonyl)hexanoic Acid Propyl Ester (10). Compound **10** was obtained by reacting benzyl ester **27** (280 mg, 0.38 mmol) according to general procedure D followed by general procedure A with amine **13** (88.0 mg, 0.38 mmol). The product was obtained (230 mg, 0.24 mmol, 63%) as a white solid after crystallization from ether/ethyl acetate (1:1). $R_f = 0.25$ (CH_2Cl_2/MeOH, 95: 5). ^{1}H NMR ($CDCl_3$/CD_3OD, 95:5, 300 MHz) δ: 0.79−0.98 (24H, m), 1.15−1.68 (32H, bm), 1.79 (1H, m), 1.92 (1H, m), 2.29−2.53 (2H, bm), 2.97 (2H, t), 3.23 (2H, t), 3.83 (2H, bm), 4.04 (2H, t), 4.35−4.46 (2H, m), 4.63 (1H, t), 7.02 (4H, d), 7.80 (4H, d). HRMS calcd for $C_{52}H_{82}N_6O_{11}Na$ $(M + Na)^+$: 989.5934. Found: 989.5923.

(*S*)-2-(2*S*-(4-(4-(((2*S*,3*S*)-1-((3*S*,4*S*)-1-(Butylamino)-3-hydroxy-6-methyl-1-oxoheptan-4-ylamino)-3-methyl-1-oxopentan-2-yl)-carbamoyl)phenoxy)benzamido)propanamido)-6-aminohexano-ic Acid Propyl Ester (9). Product **10** (40.0 mg, 0.04 mmol) was reacted under general procedure B to give compound **9** as a white solid purified by digestion with ethyl ether (35 mg, 0.04 mmol, 96%): mp 154 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 0.60−0.99 (24H, m), 1.21−1.71 (20H, bm), 1.93 (1H, m), 2.08 (2H, m), 2.75 (2H, t), 3.04 (2H, t), 3.82 (2H, m), 3.98 (2H, t), 4.27 (2H, m), 4.54 (1H, m), 7.01 (4H, d), 7.96 (4H, d). HRMS calcd for $C_{47}H_{74}N_6O_9$-Na $(M + Na)^+$: 889.54095. Found: 889.54006. HRMS calcd for $C_{47}H_{75}N_6O_9$ $(M + H)^+$: 867.5590. Found: 867.5572.

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Supporting Information Available: HPLC purity measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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