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Antiplasmodial activity of short peptide-based compounds†

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Three series of short peptide-based compounds were synthesized, which upon evaluation against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum in vitro*, produced IC_{50} values ranging between 1.4–4.7 μg mL^{-1}. Importantly, higher antimalarial activity against the drug-resistant strain of *P. falciparum* (W2) was observed for the tested peptides, indicating their potential in the treatment of drug-resistant malaria parasites. The lack of cytotoxicity in all tested peptides provides evidence of their safety profile. The selected peptides were evaluated in an enzymatic inhibitory assay against plasmepsin II, a potential target for antiplasmodial activity, also indicated from the results of the molecular docking studies.

Introduction

With approximately 219 million cases and 660 000 attributed deaths reported globally in 2013, malaria is one of the most severe infectious diseases in the world.[1] In Africa alone, more than one million children under the age of five die from malaria each year, and it remains inextricably linked with the poverty. The unavailability of a vaccine and the spread of drug resistance over the past 15–20 years have led to a dramatic decline in the efficacy of the most affordable drugs.[2] Human infection can be caused by four major species of the malaria parasite, *i.e.*, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, of which *P. falciparum* is responsible for more than 95% of malaria-related morbidity and mortality.[3] *P. falciparum*, the most lethal of the four parasites infecting humans, is increasingly becoming resistant to many of the current frontline antimalarial drugs including, chloroquine, quinine, artemisinin, and sulfadoxime/pyrimethamine.[4] This has been recognized as a major health concern, highlighting the urgent need for new treatment paradigms and for new efficacious drugs to combat this disease.

The life cycle of *Plasmodium* parasite starts with the invasion into human erythrocytes and consumes up to 75% of the hemoglobin present.[5] The digestive vacuoles are acidic single-membrane organelles with an internal pH between 4 and 5.4. Four of the ten aspartic proteases found in the genome, have been localized to the acidic food vacuole of the parasite, namely plasmepsin I, II, IV, and HAP, a histo-aspartic protease.[6] Plm I and Plm II make the first strategic cleavage of hemoglobin between Phe33 and Leu34 of the R-chain, resulting in protein unfolding and release of the heme moiety. Subsequent degradation steps are catalyzed by the cysteine protease falcipain, the metalloprotease falcilysin, and cytoplasmic aminopeptidases (Fig. 1). The amino acids derived from hemoglobin degradation are utilized by the parasite as an energy source.[7] In addition, the clearance of hemoglobin from the erythrocyte provides space for the growing parasite.

The inhibition of any of these enzymes leads to the starvation of the parasite and has been proposed as a viable strategy for drug development.[8] Plm II is the most thoroughly studied

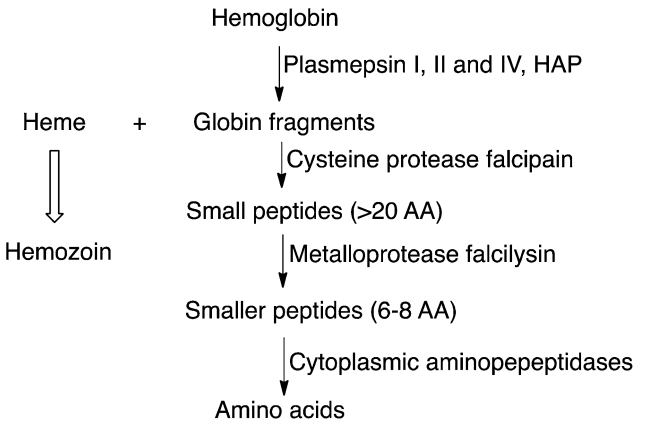

Fig. 1 Degradation of hemoglobin by plasmepsin and associated proteases.

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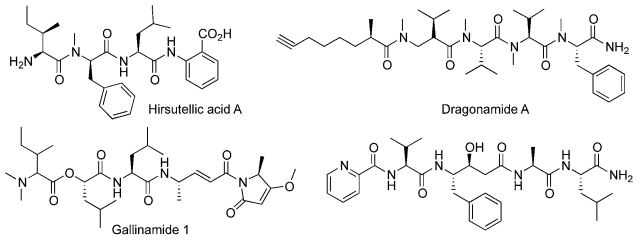


Fig. 2 Known antiplasmodial peptides.

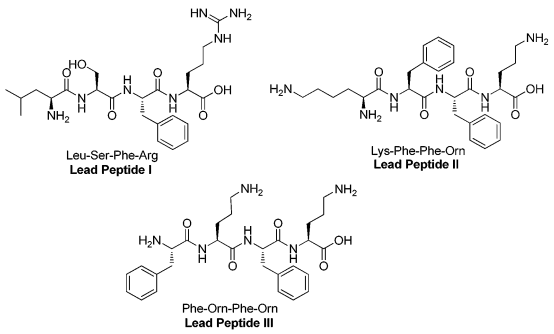


Fig. 3 Structure of the lead compounds.

enzyme among the aspartyl proteases of *Plasmodium*.[9] In 1996, the crystal structure of plasmepsin II complexed with pepstatin A was published.[10] It is made up of 329 amino acids folded into two topologically similar N- and C-terminal domains and may be a suitable molecular target for future antimalarial drugs. In the light of availability of the crystal structure of Plm II, a number of inhibitors have been reported in the past.[11]

In this paper we report the synthesis, biological evaluation against *P. falciparum* growth, enzymatic inhibition assay and molecular docking studies against Plm II of three series of short peptide-based inhibitors.

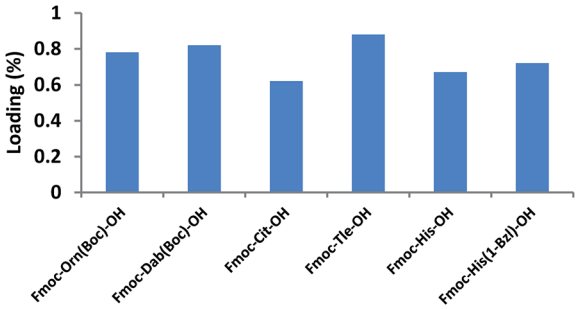


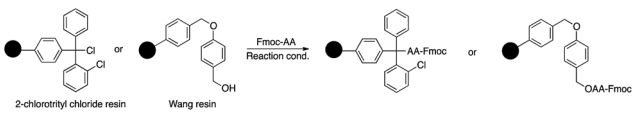
Fig. 4 Loading of various amino acids on to the 2-chlorotrityl chloride resin under optimized conditions.

Results and discussion

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.[12] In search of peptide-based inhibitors of *P. falciparum*, we came across a number of natural and synthetic peptides having diverse sequences. Two long linear peptides psalmopeotoxin I (33 amino acids) and psalmopeotoxin II (27 amino acids) were isolated from the venom of tarantula having IC_{50} values of 1.59 μM and 1.15 μM against *P. falciparum*.[13] Antimicrobial peptide dermaseptin S4 derivatives were also identified for anti-plasmodial activity. In a study, it has been observed that the truncated dermaseptin S4 from several portions, and their shorter sequences retained the antiplasmodial activity.[14]

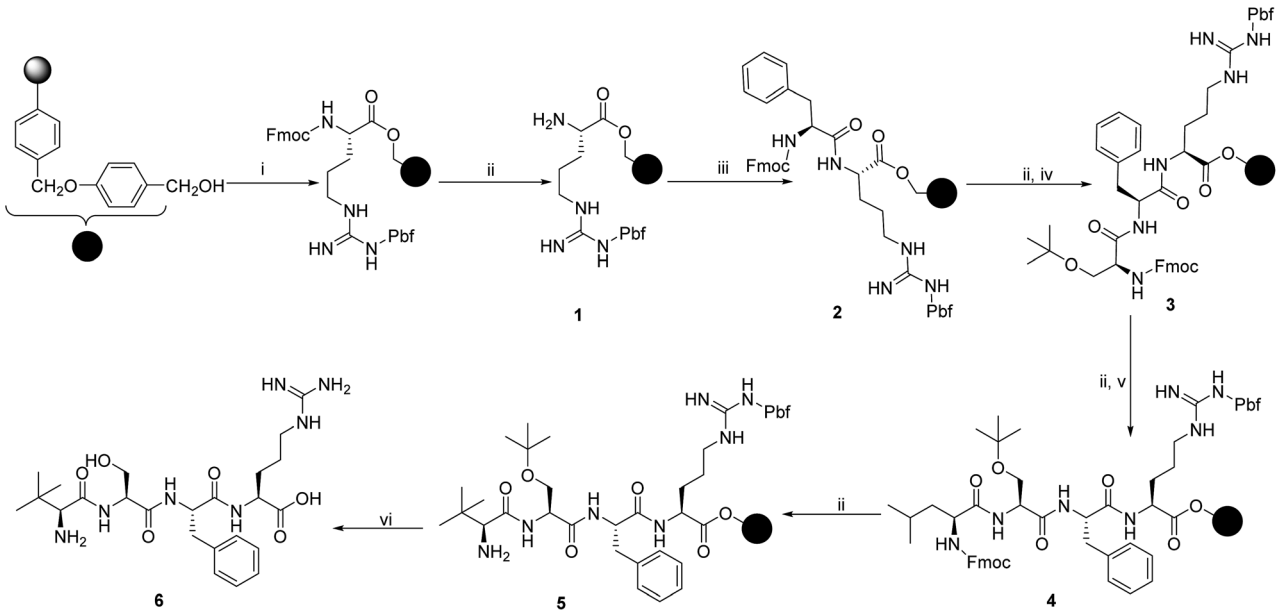
Several natural peptides were isolated from different resources and exhibit activity against *P. falciparum* with a

Table 1 Optimization of loading of resin under various sets of reaction conditions[a]



Entry no.	Base	Solvent	Equiv. of base	Time (h)	Loading 2-CTC[a]	Loading Wang[b]
1	DMAP	DCM	3	6–9	0.47	0.40
2	TEA	DCM	3	6–9	0.24	0.28
3	DBU	DCM	3	6–9	0.31	0.25
4	DIEA	DCM	3	6–9	0.56	0.48
5	Collidine	DCM	3	6–9	0.38	0.30
6	DIEA	1,4-Dioxane	3	6–9	0.42	0.36
7	DIEA	THF	3	6–9	0.43	0.34
8	DIEA	DCE	3	6–9	0.54	0.44
9	DIEA	DMF	3	6–9	0.69	0.53
10	DIEA	NMP	3	6–9	0.43	0.35
11	DIEA	DMF	3	6–9	0.78	0.59

[a] Reaction conditions: Fmoc-Orn(Boc)-OH (3 equiv.), base (3 equiv.), solvent (2 mL). [b] Fmoc-Orn(Boc)-OH (3 equiv.), TBTU (3 equiv.), solvent (2 mL).



Reagents and conditions: (i) Fmoc-Arg(Pbf)-OH, TBTU, DIEA, DMF, 9 h; (ii) 20% piperdine in DMF, 15 min; (iii) Fmoc-Phe-OH, TBTU, DIEA, DMF, 2 h; (iv) Fmoc-Ser(*t*-Bu)-OH, TBTU, DIEA, DMF, 2 h; (v) Fmoc-Tle-OH, TBTU, DIEA, DMF, 2 h; (vi) triisopropylsilane, TFA, H_2O, 2.5 h.

Scheme 1 Generalized scheme for solid phase peptide synthesis.

range of IC_{50} values of 4–9 μM (Fig. 2), but produced cytotoxicity in Vero cells.[15–19] As shown in Fig. 2, tetrapeptide hirsutellic acid A[15] having amino acids isoleucine (I, Ile), leucine (L, Leu) and *N*-methylphenylalanine (N-Me-Phe) exhibit IC_{50} value of 8.0 μM against *P. falciparum*, and was non-cytotoxic to Vero cells at a concentration of 95 μM. Other antiplasmodial peptides isolated from natural resources are dragonamide A[16] and gallinamide 1,[17] that exhibit IC_{50} and cytotoxicity values of 7.7 μM and 67.8 μM (dragonamide), and 8.4 μM and 10.4 μM (gallinamide). Synthetic peptides were also reported in the literature having potent antimalarial activity with best exhibiting IC_{50} value of 0.56 nM.[18,19] A key structural feature in these sequences is the presence of hydrophobic amino acids such as Phe, Leu, Valine (V, Val) and Ile. Also the presence of a hydroxyl or hydroxyl-like moiety is observed that plausibly coordinates to the catalytic dyad of an aspartic protease as described in the earlier reports.[20]

In a recent study, E. Candolfi and co-workers have identified a small peptide fragment Leu-Ser-Phe-Arg from various chromogranins sequences.[21] This small peptide inhibits 50% growth of *P. falciparum* strain 3D7 at 20 μM and satisfies the minimum pharmacophoric requirement for activity having cationic amino acid Arg, hydrophobic amino acids Leu and Phe, and also the hydroxyl group-containing amino acid serine (Fig. 3). In another independent study, two tetrapeptides having sequence Phe-Orn-Phe-Orn and Lys-Phe-Phe-Orn showed antimalarial activity with IC_{50} values of 3.31 and 2.57 μM, respectively (Fig. 3).[22] Herein, compounds were designed on the concept of bioisosteric replacement, which is an established strategy to have been extensively used to optimize lead compounds in the peptide-based drug discovery.[23,24]

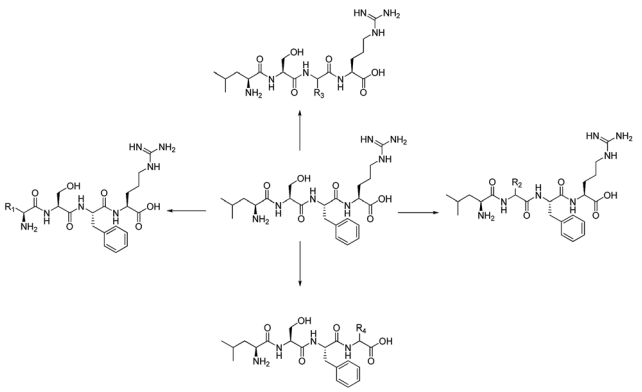


Fig. 5 Representative example of peptide modification at various positions.

Chemistry

To synthesize desired peptides, we first optimized the loading of resin in our laboratory.[25,26] The optimization of the loading of resin for different amino acids (AAs) was performed under a variety of reaction conditions. The loading capacity was determined by standard protocol assay.[27]

In order to optimize the loading on 2-chlorotrityl chloride (2-CTC) and Wang resins, first we screened a number of homogenous bases. As shown in Table 1, the most promising results were obtained with *N*,*N*-diisopropylethylamine (DIEA) in DCM (dichloromethane) (entry no. 4). In case of DMAP (4-dimethylaminopyridine) (entry no. 1), the loading is somewhat promising, whereas with other bases such as TEA (triethylamine),

Table 2 *In vitro* antimalarial activities of peptides (series 1)[a]

Peptide	Substitution	Sequence	*P. falciparum* (D6)		*P. falciparum* (W2)	
			IC_{50} (μg mL^{-1})	SI[b]	IC_{50} (μg mL^{-1})	SI[b]
Modification at R_1						
7	Leu	L-S-F-R	NA	–	NA	–
6	Tle	Tle-S-F-R	3.6	>2.7	3.2	>3.1
8	Ile	I-S-F-R	NA	–	NA	–
9	Val	V-S-F-R	NA	–	NA	–
10	Nle	Nle-S-F-R	4.2	>2.3	4.0	>2.5
11	homoLeu	hLeu-S-F-R	4.0	>2.5	3.8	>2.6
12	Nval	Nval-S-F-R	4.0	>2.5	3.6	>2.7
13	Aib	Aib-S-F-R	3.0	>3.3	3.0	>3.3
14	D-Leu	D-L-S-F-R	NA	–	NA	–
Modification at R_2						
15	Tyr	L-Y-F-R	NA	–	NA	–
16	Thr	L-T-F-R	NA	–	NA	–
17	homoSer	L-hSER-F-R	NA	–	NA	–
18	N-Me-Ser	L-NMeSer-F-R	NA	–	NA	–
19	D-Ser	L-D-S-F-R	2.1	>4.7	1.8	>5.5
Modification at R_3						
20	2-Nal	L-S-2-Nal-R	2.4	>4.1	1.8	>5.5
21	Phe(4-Me)	L-S-F(4-Me)-R	NA	–	NA	–
22	Phe(4-Br)	L-S-F(4-Br)-R	3.0	>3.3	2.0	>5
23	Phe(4-Cl)	L-S-F(4-Cl)-R	2.4	>4.1	1.7	>5.8
24	Phe(4-NH_2)	L-S-F(4-NH_2)-R	2.0	>5	1.6	>6.2
25	Phe(4-*t*-Bu)	L-S-F(4-*t*-Bu)-R	3.8	>2.6	3.2	>3.1
26	homoPhe	L-S-hPhe-R	NA	–	NA	–
27	Cha	L-S-F-R	3.2	>3.1	3.0	>3.3
28	D-Phe	L-S-D-F-R	2.1	>4.7	1.8	>5.5
Modification at R_4						
29	Cit	L-S-F-Cit	NA	–	NA	–
30	Orn	L-S-F-Orn	NA	–	NA	–
31	Dab	L-S-F-Dab	NA	–	NA	–
32	His(1-Bzl)	L-S-F-H(1-Bzl)	NA	–	NA	–
33	Lys	L-S-F-K	2.0	>5	1.7	>5.8
34	His	L-S-F-H	NA	–	NA	–
35	D-Arg	L-S-F-D-R	NA	–	NA	–
36	D-Arg*	D-L-D-S-D-F-D-R	1.9	>5.2	1.7	>5.8

[a] IC_{50} are the sample concentration that kills 50% cells compared to vehicle control; NC, not cytotoxic up to (10 μg mL^{-1}); NA, not active; "–", not calculated. Chloroquine: IC_{50} = 0.014 μg mL^{-1}, SI = 714 (D6 clone); IC_{50} = 0.1 μg mL^{-1}, SI = 100 (W2 clone); artemisinin: IC_{50} = 0.015 μg mL^{-1}, SI = 666 (D6 clone); IC_{50} = 0.009 μg mL^{-1}, SI = 1111 (W2 clone). [b] Selectivity index (SI) is the ratio of IC_{50} in Vero cells to IC_{50} in *P. falciparum* (D6 or W2). * Containing all D-amino acids.

DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and collidine, the loading was low (entry nos 2, 3 and 5). After optimization of the base, a number of non-polar solvents like THF (tetrahydrofuran), DCE (1,2-dichloroethene) and polar solvents such as NMP (*N*-methyl-2-pyrrolidone) and DMF (dimethylformamide) were screened. DMF (entry no. 9) appears to be best among all the screened solvents. After optimization of base and solvent, we performed the double coupling of amino acids in the presence of DIEA as a base, and DMF as solvent. We found that this condition gives the best loading results (entry no. 11), among all the experiments performed.

The extent of racemization during the optimized loading procedure was determined by preparing the dipeptides, which upon HPLC analysis was found to be less than 0.1%. After optimal conditions were defined for the cross-linking of the resin into amino acids, desired amino acids was preloaded on the solid support (Fig. 4). The peptides for the desired study were synthesized using the Fmoc protocol of the solid phase peptide synthesis (SPPS) on a fully automated peptide synthesizer (Aapptec, Focus XC) as shown in the Scheme 1.

A library of peptides was synthesized based on lead peptides I, II and III. We concentrated our efforts on a systematic amino acid replacement strategy without increasing the peptide backbone length. The first site examined was the N-terminus amino acid in the lead peptide I. Leucine was replaced with other hydrophobic and sterically hindered amino acids at the R_1 position to see the effect of its replacement on the antimalarial activity of the peptides keeping the other three amino acids

Table 3 *In vitro* antimalarial activities of peptides (series 2)[a]

Peptide	Substitution	Sequence	*P. falciparum* (D6)		*P. falciparum* (W2)	
			IC_{50} (μg mL^{-1})	SI[b]	IC_{50} (μg mL^{-1})	SI[b]
Modification at R_1						
37	Lys	K-F-F-O	2.0	>5	1.6	>6.2
38	Orn	O-F-F-O	NA	—	NA	—
39	Dab	Dab-F-F-O	NA	—	NA	—
40	Arg	R-F-F-O	2.6	>3.8	1.8	>5.5
41	Cit	Cit-F-F-O	NA	—	NA	—
42	His	H-F-F-O	NA	—	NA	—
43	D-Lys	D-K-F-F-O	NA	—	NA	—
Modification at R_2						
44	Phe(4-NH_2)	K-F(4-NH_2)-F-O	1.6	>6.2	1.4	>7.1
45	Cha	K-Cha-F-O	NA	—	NA	—
46	Phe(4-Cl)	K-F(4-Cl)-F-O	1.6	>6.2	1.4	>7.1
47	Phe(4-*t*-Bu)	K-F(4-*t*-Bu)-F-O	NA	—	NA	—
48	homoPhe	K-hPhe-F-O	NA	—	NA	—
49	D-Phe	K-D-F-F-O	NA	—	NA	—
Modification at R_3						
50	Phe(4-NH_2)	K-F-F(4-NH_2)-O	NA	—	NA	—
51	Cha	K-F-Cha-O	NA	—	NA	—
52	Phe(4-Cl)	K-F-F(4-Cl)-O	NA	—	NA	—
53	Phe(4-*t*-Bu)	K-F-F(4-*t*-Bu)-O	2.0	>5	1.6	>6.2
54	homoPhe	K-F-hPhe-O	NA	—	NA	—
55	D-Phe	K-F-D-F-O	NA	—	NA	—
Modification at R_4						
56	Arg	K-F-F-R	2.0	>5	1.6	>6.2
57	His	K-F-F-H	3.2	>3.1	3.0	>3.1
58	Cit	K-F-F-Cit	NA	—	NA	—
59	Dab	K-F-F-Dab	2.6	>3.8	1.8	>5.5
60	Lys	K-F-F-K	2.6	>3.8	1.8	>5.5
61	D-Orn	K-F-F-D-O	NA	—	NA	—
62	D-Orn*	D-K-D-F-D-F-D-O	NA	—	NA	—

[a] IC_{50} are the sample concentration that kills 50% cells compared to vehicle control; NC, not cytotoxic up to (10 μg mL^{-1}); NA, not active; "—", not calculated. Chloroquine: IC_{50} = 0.014 μg mL^{-1}, SI = 714 (D6 clone); IC_{50} = 0.1 μg mL^{-1}, SI = 100 (W2 clone); artemisinin: IC_{50} = 0.015 μg mL^{-1}, SI = 666 (D6 clone); IC_{50} = 0.009 μg mL^{-1}, SI = 1111 (W2 clone). [b] Selectivity index (SI) is the ratio of IC_{50} in Vero cells to IC_{50} in *P. falciparum* (D6 or W2). * Containing all D-amino acids.

intact (Fig. 5). Next, to explore the importance of polar amino acid serine (Ser) at the position 2; we replaced it with other hydrophilic amino acids (containing a hydroxyl group) at the R_2 position in lead compound I. Similarly, we systematically replaced the phenylalanine (Phe) residue at the position 3 and the C-terminus amino acid arginine in the lead peptide I with its bioisosteric counterparts. Similar strategy was adopted to synthesize peptides of series 2 and 3.

Antiplasmodial activity

All synthesized peptides were tested *in vitro* for antimalarial activity against chloroquine sensitive (D6) and resistant *P. falciparum* strains (W2) and results are shown in Tables 2–4. *In vitro* antimalarial activity was determined on the basis of plasmodial LDH activity, and expressed as IC_{50} values *versus* chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum*.[28,29]

Among peptides **6–14**, which represent replacement at the R_1 position, peptide **13** (R_1 = Aib) produced promising activity against D6 and W2 strains with IC_{50} values of 3.0 μg mL^{-1}. While analogs **6** (R_1 = Tle), **10** (R_1 = Nle), **11** (R_1 = homoLeu) and **12** (R_1 = Nval) showed activity against D6 and W2 strains with IC_{50} values of 3.6 and 3.2 μg mL^{-1}, 4.2 and 4.0 μg mL^{-1}, 4.0 and 3.8 μg mL^{-1}, 4.0 and 3.6 μg mL^{-1}, respectively. The peptide analog **14** containing all D-amino acids was inactive at the highest tested concentration of 10 μg mL^{-1}.

Peptides **15–19** represents replacement at the R_2 position in lead peptide I. Of these, peptide **19** (R_2 = D-Ser) exhibited encouraging activity against D6 and W2 strains with IC_{50} values of 2.1 and 1.8 μg mL^{-1}, respectively. Preliminary structure activity observations indicate that only Ser containing peptide has demonstrated antiplasmodial activity from this series of compounds. In general, replacement of Ser residue at the 2 position in the lead compound I results in inactive peptides. Peptides **20–28**, represent replacement of Phe residue at the

Table 4 *In vitro* antimalarial activities of peptides (series 3)[a]

Peptide	Substitution	Sequence	*P. falciparum* (D6)		*P. falciparum* (W2)	
			IC_{50} (μg mL^{-1})	SI[b]	IC_{50} (μg mL^{-1})	SI[b]
Modification at R_1						
63	Phe	F-O-F-O	4.0	>2.5	4.0	>2.5
64	Phe(4-Cl)	F(4-Cl)-O-F-O	2.0	>5	1.6	>6.2
65	Phe(4-NH_2)	F(4-NH_2)-O-F-O	2.6	>3.8	1.8	>5.5
66	Phe(4-*t*-Bu)	F(4-*t*-Bu)-O-F-O	4.0	>2.5	4.0	>2.5
67	Cha	Cha-O-F-O	NA	—	NA	—
68	homoPhe	hPhe-O-F-O	NA	—	NA	—
69	D-Phe	D-F-O-F-O	NA	—	NA	—
Modification at R_2						
70	Lys	K-O-F-O	NA	—	NA	—
71	His	H-O-F-O	4.7	>2.1	4.2	>2.1
72	Arg	R-O-F-O	NA	—	NA	—
73	Cit	Cit-O-F-O	NA	—	NA	—
74	Dab	Dab-O-F-O	3.2	>3.1	3.0	>3.3
75	D-Orn	F-D-O-F-O	NA	—	NA	—
Modification at R_3						
76	Phe(4-Cl)	F-O-F(4-Cl)-O	2.0	>5	1.6	>6.2
77	Phe(4-NH_2)	F-O-F(4-NH_2)-O	2.6	>3.8	1.8	>5.5
78	Phe(4-*t*-Bu)	F-O-F(4-*t*-Bu)-O	1.6	>6.2	1.4	>7.1
79	Cha	F-O-Cha-O	NA	—	NA	—
80	homoPhe	F-O-hPhe-O	NA	—	NA	—
81	D-Phe	F-O-D-F-O	NA	—	NA	—
Modification at R_4						
82	Arg	F-O-F-R	1.6	>6.2	1.4	>7.1
83	His	F-O-F-H	NA	—	NA	—
84	Lys	F-O-F-K	2.6	>3.8	1.8	>5.5
85	Dab	F-O-F-Dab	2.6	>3.8	1.8	>5.5
86	Cit	F-O-F-Cit	NA	—	NA	—
87	D-Orn	F-O-F-D-O	NA	—	NA	—
88	D-Orn*	D-F-D-O-D-F-D-O	NA	—	NA	—

[a] IC_{50} are the sample concentration that kills 50% cells compared to vehicle control; NC, not cytotoxic up to (10 μg mL^{-1}); NA, not active; "—", not calculated. Chloroquine: IC_{50} = 0.014 μg mL^{-1}, SI = 714 (D6 clone); IC_{50} = 0.1 μg mL^{-1}, SI = 100 (W2 clone); artemisinin: IC_{50} = 0.015 μg mL^{-1}, SI = 666 (D6 clone); IC_{50} = 0.009 μg mL^{-1}, SI = 1111 (W2 clone). [b] Selectivity index (SI) is the ratio of IC_{50} in Vero cells to IC_{50} in *P. falciparum* (D6 or W2). * Containing all D-amino acids.

3 position in the lead peptide with its aromatic hydrophobic counterparts. Analog **24** [R_3 = Phe(4-NH_2)] produced promising activity against D6 and W2 strains with IC_{50} values of 2.0 and 1.6 μg mL^{-1}, respectively. While, analogs **20** (R_3 = 2-Nal), **23** [R_3 = Phe(4-Cl)] and **28** (R_3 = D-Phe) also showed encouraging activities against D6 and W2 strains by exhibiting IC_{50} values of 2.4 and 1.8 μg mL^{-1}, 2.4 and 1.7 μg mL^{-1} and 2.1 and 1.8 μg mL^{-1}, respectively.

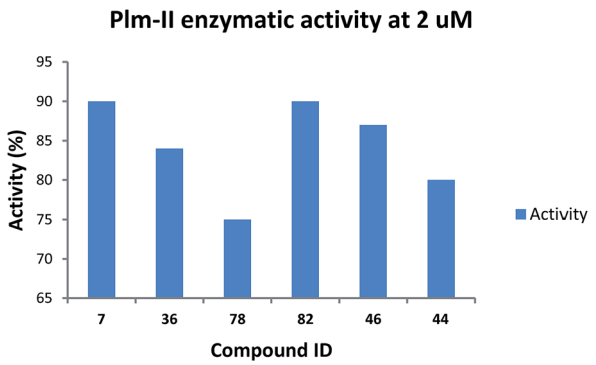


Fig. 6 PLM II assay of selected peptides.

The analogs **22** [R_3 = Phe(4-Br)], **25** [R_3 = Phe(4-*t*-Bu)] and **27** (R_3 = Cha) also showed comparable activities against D6 and W2 strains with IC_{50} values of 3.0 and 2.0 μg mL^{-1}, 3.8 and 3.2 μg mL^{-1} and 3.2 and 3.0 μg mL^{-1}, respectively. Peptides **29–36** represent the replacement of R_4 position with hydrophilic amino acids. Analog **36** containing all D-amino acids showed the highest potency against D6 and W2 strains with IC_{50} values of 1.9 and 1.7 μg mL^{-1}, respectively. While analogs **33** (R_4 = Lys) showed good activity against D6 and W2 strains with IC_{50} values of 2.0 and 1.7 μg mL^{-1}, respectively. In general, peptides have displayed higher potency against drug-resistant W2 strain (Table 2).

The activities of peptides **37–62** (series 2) are shown in Table 3. In the case of peptides **37–43**, in which N-terminus

Table 5 Analysis of 10 co-crystallized structures to identify critical amino acids necessary for the plasmepsin inhibition[a]

PDB ID	Resolution (Å)	Ligand	K_i/IC_{50} (nM)	Similarity with peptide ligand	Asp34	Val78	Asn76	Leu131	Ser79	Ser218	Tyr192	Gly216	Phe111
2BJU	1.56	IH4	34	0.08							pi		pi
2IGX	1.70	A1T	54	0.08							pi		pi
1LF2	1.80	R37	30	0.21	H	H		H	H				
1LEE	1.90	R36	18	0.21	H	H	H		H + pi				
1W6H	2.24	TIT	0.5–2.2	0.20	H	H	H		H	H	H		pi
1XE5	2.40	5FE		0.13	H	H	H		H	H	H	H	
2IGY	2.60	A2T	113	0.09						H			pi
1ME6	2.70	IVS		0.12	H				H	H			
1LF3	2.70	EH5	100	0.15	H	H			H	H		H	
1XE6	2.80	5FP		0.17		H			H	H	H		

[a] H, hydrogen bond donor or acceptor; pi, π–π interaction.

amino acid Lys is replaced, analog **37** (R_1 = Lys) produced activity against D6 and W2 strains and displayed IC_{50} values of 2.0 and 1.6 μg mL^{-1}, respectively. While analog **40** (R_1 = Arg) showed good activity against D6 and W2 strains with IC_{50} values of 2.6 and 1.8 μg mL^{-1}, respectively. All other analogs of this series were inactive against D6 and W2 strains. The peptide analog **43** containing D-Lys was inactive at the highest test concentration of 10 μg mL^{-1}. For peptides **44–49**, in which substitutions at the 2-position of lead peptide II were carried out, analog **44** [R_2 = Phe(4-NH_2)] and **46** [R_2 = Phe(4-Cl)] were the most potent peptides against D6 and W2 strains, and produced IC_{50} values of 1.6 and 1.4 μg mL^{-1}, respectively. It was observed that replacement of Phe with unnatural side-chain containing residues Cha (**45**) and homoPhe (**48**) results in total loss of activity. In the case of replacement of Phe at the position 3 with Phe(4-*t*-Bu) (**53**) promising activity against D6 and W2 strains with IC_{50} values of 2.0 and 1.6 μg mL^{-1}, respectively was observed. The remaining substitution at this position proves to be non-consequential for the activity. The peptides also produced selectivity index between >7.1 to 3.1 for both tested strains. Replacement at C-terminus with other hydrophilic amino acids, produced **56** (R_4 = Arg) exhibiting encouraging activity against D6 and W2 strains with IC_{50} values of 2.0 and 1.6 μg mL^{-1}, respectively. While peptides **59** (R_4 = Dab) and **60** (R_4 = Lys) showed activity against D6 and W2 strains with IC_{50} values of 2.6 and 1.8 μg mL^{-1}, respectively. Analog **57** (R_4 = His) also exhibited modest activity against D6 and W2 strains by producing IC_{50} values of 3.2 and 3.0 μg mL^{-1}, respectively.

The antiplasmodial activities of peptides of series 3 are presented in Table 4. The peptides **64** [R_1 = Phe(4-Cl)] and **65** [R_1 = Phe(4-NH_2)] exhibited promising activity against D6 and W2 strains with IC_{50} values of 2.0 and 1.6 μg mL^{-1} and 2.6 and 1.8 μg mL^{-1}, respectively. While analogs **63** (R_1 = Phe) and **66** [R_1 = Phe(4-*t*-Bu)] showed activity against D6 and W2 strains with IC_{50} values of 4.0 μg mL^{-1}, respectively. For peptides **70–75**, in which substitutions at the position 2 of lead peptide III were carried out, analog **74** (R_2 = Dab) exhibited modest activity against D6 and W2 strains with IC_{50} values of 3.2 and 3.0 μg mL^{-1}, respectively. While analog **71** (R_2 = His) also showed activity against D6 and W2 strains with IC_{50} values of 4.7 and 4.2 μg mL^{-1}, respectively.

For peptides **76–81**, in which Phe at 3 position is replaced, peptide analog **78** [R_3 = Phe(4-*t*-Bu)] was the most promising peptide and produced IC_{50} values of 1.6 and 1.4 μg mL^{-1}, respectively against D6 and W2 strains (Table 4, series 3). While analog **76** [R_3 = Phe(4-Cl)] showed encouraging activity against D6 and W2 strains with IC_{50} values of 2.0 and 1.6 μg mL^{-1}, respectively. Analog **77** [R_3 = Phe(4-NH_2)] also showed modest IC_{50} values of 2.6 and 1.8 μg mL^{-1}, respectively against D6 and W2 strains. Substitutions at the C-terminus in lead peptide III gave seven analogs **82–88**. Analog **82** (R_4 = Arg) exhibiting promising activity against D6 and W2 strains with IC_{50} values of 1.6 and 1.4 μg mL^{-1}, respectively. Analogs **84** (R_4 = Lys) and **85** (R_4 = Dab) showed modest activity against D6 and W2 strains with IC_{50} values of 2.6 and 1.8 μg mL^{-1}, respectively. The peptides belonging to this series, in agreement with the observation made for most of the peptide analogs screened were found more effective against drug-resistant W2 strain of *P. falciparum*.

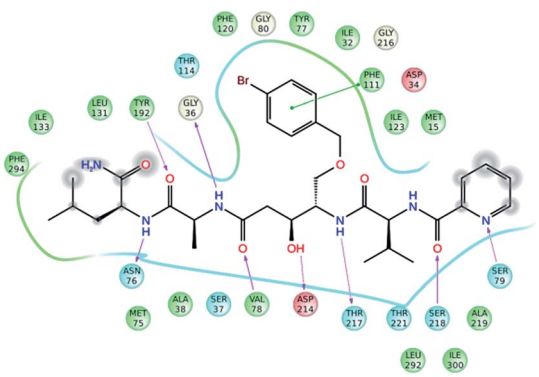


Fig. 7 The docking pose of co-crystallized ligand into the active site of plasmepsin II protein (PDB ID: 1W6H).

Cytotoxicity evaluation

All synthesized peptides were also evaluated for cytotoxicity in mammalian cell line to determine their safety profile. The *in*

Table 6 The docking pose analysis of synthesized plasmepsin II inhibitors[a]

Peptide	PSA	VAL78	SER79	THR114	TYR192	ASP34	GLU36	ASN76	ASP130	LEU131	ASP214	GLY216	THR217	SER218
63	202.66	1	1		1	1		1			1			
64	202.66					1					1	1	1	
65	228.68	1			1	1	1		1	1	1			
66	202.66		1	1								1	1	
76	202.66	1	1		1	1					1			
77	228.68	1			1	1	1	1		1	1			
78	202.66	1			1	1	1	1			1			
71	205.31		1			1					1	1		1
74	202.66	1									1		1	
84	202.66	1						1			1	1		
85	202.66			1		1					1			
57	205.32	1						1			1			1
37	202.66					1					1		1	1
53	202.66						1				1	1	1	1
46	202.66					1					1		1	1
44	228.68		1			1					1	1		1
59	202.66	1	1	1		1		1			1		1	
60	202.66		1			1					1		1	1
Co-crystal	106	1	1		1		1	1			1		1	1

[a] 1, presence of hydrogen bond interaction.

vitro cytotoxicity of analogs was determined against VERO noncancerous mammalian cells (obtained from ATCC, American Type Culture Collection) by neutral red assay.[29] None of the peptides exhibited cytotoxicity at the tested concentration of 10 μg mL^{-1}.

Enzyme inhibition assay against PLM II

Six compounds from the series were tested in enzymatic assays against the catalytic activity of plasmepsin-II from *P. falciparum.* The compounds were first dissolved in DMSO and then diluted into buffered solutions at pH 4.5 for assay. The results are shown in the Fig. 6 below. The compounds were assayed at a concentration of 2 μM. The tests were done twice and the % activity values given above are averages of the duplicate determinations. The maximum inhibition was 25% for peptide **78**.

Molecular modeling

A large number of crystal structures of plasmepsin II are reported in the protein data bank (PDB). From the available eighteen crystal structures of plasmepsin II (PLM II), ten PLM II and inhibitor complex crystal structures were analyzed to

Table 7 The IC_{50} value, docking score, glide score, glide emodel and glide energy for the synthesized plasmepsin II inhibitors

Peptide	*P. falciparum* D6 IC_{50} (μg mL^{-1})	*P. falciparum* W2 IC_{50} (μg mL^{-1})	Docking score	Glide score	Glide emodel	Glide energy
78	1.6	1.4	−9.65355	−9.93805	−130.391	−70.5439
46	1.6	1.4	−10.4831	−10.6687	−138.429	−68.6636
44	1.6	1.4	−10.1949	−10.3805	−135.495	−70.7052
64	2.0	1.6	−8.44164	−8.72614	−112.604	−61.9412
76	2.0	1.6	−11.0705	−11.355	−149.902	−76.0211
37	2.0	1.6	−9.08432	−9.86242	−119.042	−64.7812
53	2.0	1.6	−10.0685	−10.2541	−117.491	−65.8653
65	2.6	1.8	−9.29018	−9.57468	−127.521	−67.3476
77	2.6	1.8	−9.28766	−9.57216	−117.716	−64.1044
84	2.6	1.8	−9.25124	−9.53574	−116.79	−68.161
85	2.6	1.8	−8.70013	−8.98463	−118.902	−60.6688
59	2.6	1.8	−9.69515	−9.88075	−136.38	−70.5735
60	2.6	1.8	−9.58133	−9.76693	−120.921	−69.2143
74	3.2	3.0	−9.12786	−9.43276	−120.271	−66.0703
57	3.2	3.0	−9.91432	−11.063	−147.77	−80.2347
63	4.0	4.0	−9.31489	−9.89719	−126.601	−70.6532
66	4.0	4.0	−8.80126	−9.08576	−106.702	−61.1545
71	4.7	4.2	−9.17562	−9.86112	−132.68	−69.4552

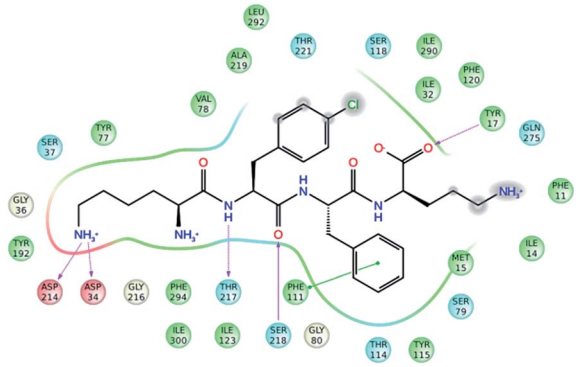


Fig. 8 The docking pose of most potent synthesized inhibitor (**46**) into the active site of plasmepsin II protein (PDB ID: 1W6H).

identify critical amino acids, necessary for the inhibition of plasmepsin. Table 5 shows hydrogen bond and π–π interaction formed by the inhibitors at the active site of PLM-II. The available crystal structures were also analyzed for their resolution and presence of similar ligands to that of the synthesized peptides. After a detailed analysis, PDB ID: 1W6H was selected for performing molecular docking analysis, as crystallized inhibitors were found to be more potent (2.2 nM) and showing high Tanimoto similarity (0.20) with the synthesized ligands.

The docking protocol was validated by re-docking of minimized conformation of co-crystallized ligand. The RMSD value between the docked conformation and the crystallized conformation was found to be 0.998 Å, which suggests that the set docking protocol is efficient to predict the bioactive conformation of the synthesized inhibitors (Fig. 7).

The Glide module failed to generate docking pose for some of the inhibitors having a total polar surface area (TPSA) higher than 228 Å (Table 6). The top ten poses were analyzed for each inhibitor. The docked pose was selected, if the peptide showed a similar pattern as that of the co-crystallized ligand. The active site residues involved in binding of synthesized ligand are shown in Table 6. Table 7 shows IC_{50} value, docking score, glide score, glide emodel and glide energy for the selected eighteen synthesized ligands. The crystal structure and molecular docking analysis reveal that the Val78, Ser79, Asp34, Asp214 and Ser218 are critical amino acids, and necessary for PLM inhibition. The docked conformation of the co-crystallized and one of the most potent synthesized peptide **46** [K-Phe(4-Cl)-F-O] is shown in Fig. 8.

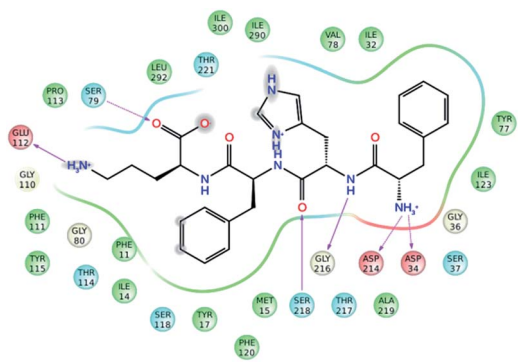


Fig. 9 The docking pose of the least potent synthesized inhibitor (**71**) into the active site of plasmepsin II protein (PDB ID: 1W6H).

The co-crystallized ligand has shown eight hydrogen bond interactions (four HBA and four HBD) with the Val78, Ser79, Tyr192, Ser218, Gly36, Asn76, Asp214 and Thr217, along with one π–π interaction with the Phe111. The peptide **46** also showed the docking score of −10.4831 and the emodel score of −138.429. The peptides **44**, **53** and **76** also produced high docking scores of −10.3805, −10.2541 and −11.355; however their emodel scores were low. Most of the synthesized peptide showed hydrogen bond interaction with carboxylic groups of Asp34 and Asp214. In case of the least potent inhibitor **71**, the hydrogen bond interactions with the critical residues Val78, Tyr192, Asn76 and Thr217 were missing, possibly resulting in low activity (Fig. 9).

Conclusions

In summary, a library of short peptide-based inhibitors was designed by applying a rational and directed approach. To synthesize peptides, we first optimized the protocol for the amino acids loading on to the Wang and 2-chlorotrityl chloride resin. The most potent peptides of this work exhibit IC_{50} value of 1.6 μg mL^{-1} and 1.4 μg mL^{-1} against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strain of *P. falciparum*. Importantly, higher antimalarial activity against the drug-resistant strain of *P. falciparum* was observed for the tested peptides, indicating their potential in the treatment of drug-resistant malaria parasite. The peptides appear to exhibit antimalarial activity by inhibiting PLM II. The lack of cytotoxicity in all tested peptides provides the evidence of their safety profile and makes these novel compounds promising for further development as antimalarial agents.

Experimental section

Materials

Amino acids, coupling reagents, *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were purchased from either Chem-Impex International or Nova Biochem (Merck Ltd.). All solvents used for synthesis were of analytical grade and were used without further purification unless otherwise stated. All other reagents were of analytical grade.

Peptide synthesis and purification

The peptides were synthesized using the Fmoc SPPS protocol on a fully automated peptide synthesizer (Aapptec, Focus XC). The coupling reactions were carried out on the 2-chlorotrityl chloride resin using Fmoc-protected amino acids (3 equiv.) and TBTU (3 equiv.) as a coupling reagent in the presence of DIEA (5 equiv.) in DMF as solvent for 120 min. N-terminus deblocking was carried out with 20% piperidine (two times) in DMF for 5 min. The peptide was washed with DMF (4 × 5 mL), and coupling and deprotection cycles were

repeated until required sequence length was obtained. The removal of peptide from the resin and deprotection of the side chain protective groups was achieved simultaneously by the reaction with a cleavage cocktail combination of 5% phenol, 2% triisopropylsilane (TIPS) and 5% H_2O in TFA for 2.5 h. After removal of the protecting groups and resin, peptides were precipitated by addition of cold diethyl ether and filtered. The crude peptides were dissolved in a mixture of H_2O–CH_3CN (1 : 1, v/v) and purified by RP-HPLC on the Shimadzu Prominence A-8 HPLC system, using a Phenomenex column (C-18, 250 × 21 mm, 10 μm), operating at 220 nm with a flow rate of 10 mL min^{-1} to afford final peptides in 90–100% purity, unless otherwise stated. The injected sample concentration was 10 mg of peptide per mL in H_2O–CH_3CN (1 : 1, v/v). The mobile phase A was 0.1% TFA in H_2O, and phase B was 0.1% TFA in CH_3CN using a linear gradient of 5 to 95% B in 40 min.

PLM II inhibitory assay

The assay for catalytic activity was developed by Westling *et al.*[30] Briefly, the gene for the proenzyme form of PLM II of *P. falciparum* was cloned into pET3a and expressed in BL21 (DE3) pLysS cells. Inclusion bodies were isolated and washed by centrifugation through a 27% sucrose gradient. The inclusion bodies were solubilized with 8 M urea and the protein refolded by dialysis *versus* a buffer of 20 mM Tris at pH 8.0. The protein was purified by anion exchange chromatography and stored at 4 °C.

The proenzyme was added to the assay buffer (sodium acetate, pH 4.5) for three-minute incubation, during which the proenzyme smoothly converts to the mature and catalytically active enzyme. The enzyme does not digest itself, so the concentration of the proenzyme added can be taken as the concentration of the enzyme produced. Aliquots of compounds to be tested were added to the enzyme for a five-minute pre-incubation to permit complex formation to proceed and then the sample was added to the assay substrate and mixed to initiate cleavage. Conversion of substrate to products was monitored by the decrease in absorbance at 300 nm in an UV/Vis spectrophotometer as a function of time. All compounds were added so that a final concentration of 2% DMSO was present and this amount was also used in the control reaction to establish full activity. It was previously determined that 2% DMSO has negligible effect on the catalytic activity.

Molecular modeling

1W6H was selected to carry out molecular docking analysis on the basis of the resolution and structural similarity with the synthesized inhibitors. The Protein Preparation Wizard and LigPrep modules support in the generation of input files, which are essential to carry out the molecular docking study. During protein preparation hydrogen atoms were added to the target, water molecules removed, the protonation states for histidine residues were optimized and the hydrogen atoms were minimized up to 0.30 Å RMSD. The grid of 10 Å was generated around the co-crystallized ligand at the active site by the Receptor Grid Generation tool. Extra precision (XP) Glide algorithm was validated to perform docking studies of the synthesized molecules, by re-docking the co-crystallized inhibitor into the grid of the target. LigPrep was used for energy minimizations of the synthesized ligands with the OPLS_2005 force field. The default settings for scaling the van der Waals radii were selected: a scaling factor of 0.8 and a partial charge cut-off of 0.25 with no constraints were defined for docking of synthesized ligands.

Abbreviations

AA	Amino acid
Abu	Aminobutyric acid
Ala	Alanine
Arg	Arginine
Cha	Cyclohexylalanine
CH_3CN	Acetonitrile
Dab	2,4-Diaminobutyric acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DCE	1,2-Dichloroethene
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
Fmoc	9-Fluorenylmethoxycarbonyl
His	Histidine
HPLC	High-performance liquid chromatography
IC_{50}	Inhibitory concentration that affords 50% inhibition of microbial growth
Ile	Isoleucine
Lys	Lysine
MIC	Minimum inhibitory concentration
Nal	1-Napthylalanine
Nle	Norleucine
NMP	*N*-Methyl-2-pyrrolidone
Orn	Ornithine
Phe	Phenylalanine
Ser	Serine
t-Bu	*tert*-Butyl
TEA	Triethylamine
TBTU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoro-borate
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
Thr	Threonine
TIPS	Triisopropylsilane
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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