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Short Rapid Communication

Search for Substrate-Based Inhibitors Fitting the S_2' Space of Malarial Aspartic Protease Plasmepsin II

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Abstract: Plasmepsin (Plm) has been identified as an important target for the development of new antimalarial drugs, since its inhibition leads to the starvation of *Plasmodium falciparum*. A series of substrate-based dipeptide-type Plm II inhibitors containing the hydroxymethylcarbonyl isostere as a transition-state mimic were synthesized. The general design principle was provision of a conformationally restrained hydroxyl group (corresponding to the set residue at the P_2' position in native substrates) and a bulky unit to fit the S_2' pocket. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimalarial drug; aspartic protease inhibitor; plasmepsin; transition-state mimic; allophenylnorstatine

INTRODUCTION

Malaria parasites use hemoglobin as a source of nutrients during their growth and maturation in erythrocytes. The prevention of this process is thought to be a key target for the development of a new drug against malaria. Recently, it became clear that malaria parasites encode several proteases that are essential components of their hemoglobin-degradation pathway [1]. For example, in the food vacuole of *Plasmodium falciparum*, four aspartic proteases, plasmepsin (Plm) I, II, and IV and histo-aspartic protease (HAP), whose amino acid sequences are highly conserved, are involved in the pathway [2,3]. Since it is reported that the inhibition of Plm I and II which initiate the hemoglobin-degradation leads to starvation of the parasites [4], these enzymes are the targets for the development of new antimalarial drugs and several inhibitors, including our compounds [5,6], have already been reported [7–12].

Our previous study on the development of new antimalarial drugs focused on Plm II as a target enzyme for structure-based drug design, since Plm II can be efficiently expressed in *E. coli*, and its high-resolution structure has been determined by x-ray crystallography [8]. Our HIV protease (PR) inhibitor study developed a series of substrate-based peptidomimetic inhibitors containing an allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere as an ideal

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Table 1 Substrate Cleavage Sites (*) of Plasmepsin II and HIV-1 Protease

Enzyme	Cleavage site	- P_4	P_3	P_2	P_1*P_1'	P_2'	P_3'	P_4' -
Plasmepsin II	α33/34	- Glu	Arg	Met	Phe*Leu	Ser	Phe	Pro -
	α108/109	- Leu	Leu	Val	Thr*Leu	Ala	Ala	His -
	α136/137	- Ser	Thr	Val	Leu*Thr	Ser	Lys	Tyr -
	β32/33	- Gly	Arg	Leu	Leu*Val	Val	Tyr	Pro -
HIV-1 protease	p17/p24	- Gln	Arg	Gly	Tyr*Pro	Ile	Val	Gln -
	p24/p1	- Ala	Arg	Val	Leu*Ala	Glu	Ala	Met -
	p1/p9	- Ala	Thr	Ile	Met*Met	Gln	Arg	Gly -
	p9/p6	- Pro	Gly	Asn	Phe*Leu	Gln	Ser	Arg
	TF/PR	- Ser	Phe	Asn	Phe*Pro	Gln	Ile	Thr
	PR/RT	- Thr	Leu	Asn	Phe*Pro	Ile	Ser	Pro
	RT/RN	- Ala	Glu	Thr	Phe*Tyr	Val	Asp	Gly
	RN/IN	- Arg	Lys	Ile	Leu*Phe	Leu	Asp	Gly

TF, transframe protein; PR, protease; RT, reverse transcriptase; RN, ribonuclease H; IN, integrase.

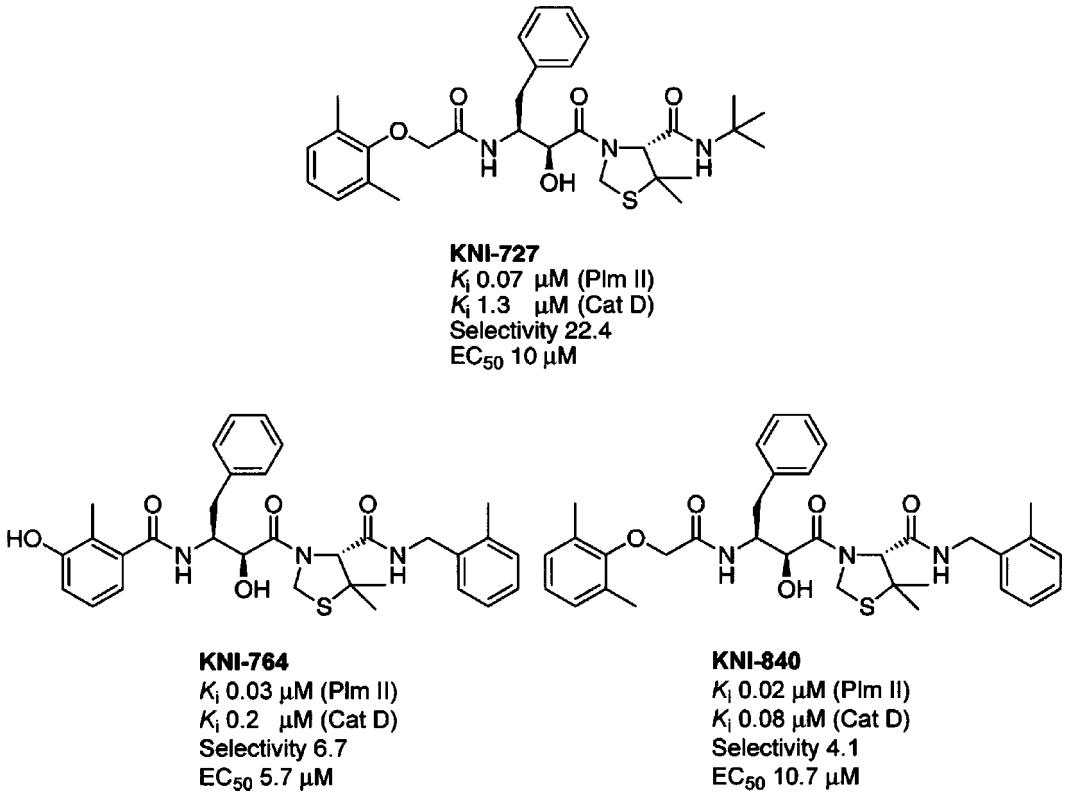


Figure 1 Structures of KNI compounds.

transition-state mimic [13–18]. Since the substrate recognition profiles of Plm I and II [19] are similar to that of HIV-1 PR (Table 1), and Phe^{33}-Leu^{34} in the α-chain which is a primary cleavage site in hemoglobin-degradation exhibits a similar structure to the Apns-containing scaffold, it was hypothesized that HIV-1 PR inhibitors would be effective against Plms, so the inhibitory activity of 12 selected HIV PR inhibitors were evaluated against Plm II [5]. As predicted, these substrate

transition-state mimic compounds are capable of inhibiting Plm II. Among them, KNI-727 [14–16], −764 [17–18] and −840 [16] which contain Apns-Dmt [Dmt = dimethylthioproline; (*R*)-5,5-dimethyl-1,3-thiazolidinecarboxylic acid] at the P_1-P_1' positions exhibited potent Plm II inhibitory activity with K_i values of 0.07, 0.03 and 0.02 μM, respectively (Figure 1). The Apns-Dmt scaffold is based on the substrate Phe-Leu (P_1-P_1') scissile site transition state; that is, Apns is the Phe-transition state mimic and Dmt is the conformational constrained cyclized Leu-mimic. Interestingly, KNI-727 also exhibited a 23-fold higher potency against Plm II compared with highly homologous human cathepsin D (Cat D). Moreover, these three compounds also exhibited antimalarial activity in cultures of red blood cells infected with *P. falciparum* with EC_{50} values of 5–10 μM (Figure 1) [5]. This result suggests that Plm II inhibitors based on the HMC-type substrate transition-state mimic are potential antimalarial drugs. In further screening, KNI-10 006, which has an (1S,2*R*)-1-amino-2-indanol at the P2′ position, was found to inhibit Plm II with a remarkably high affinity [6–20].

This paper describes the SAR of Plm II inhibitors based on the substrate transition-state mimic. Sixteen compounds (**4a**–**p**) were designed and synthesized in which the P_2' position of KNI-727 is modified (Figure 2). Since native substrates contain a Ser residue at the P_2' position (Table 1), a hydroxyl group was introduced with a fixed conformation and a bulky unit to fit the space of the S_2' pocket of Plm II. The enzyme inhibitory activities of these synthetic compounds were evaluated.

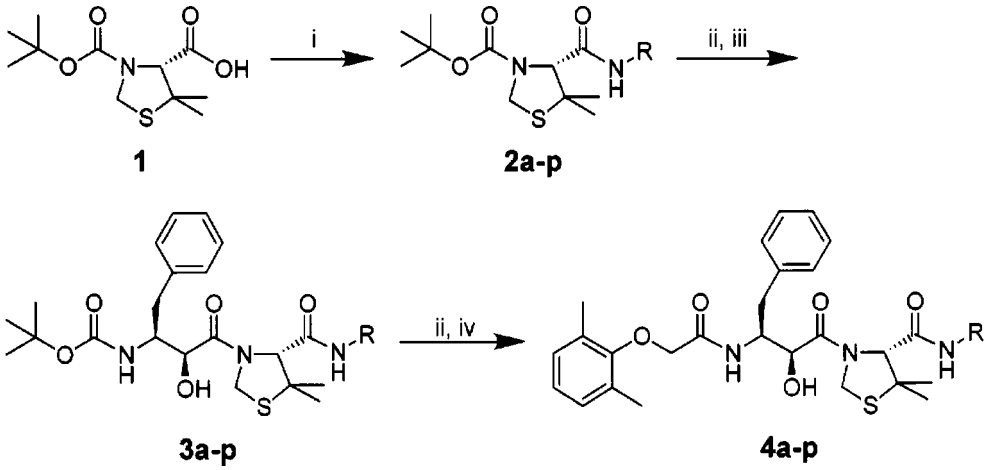
MATERIALS AND METHODS

Synthesis

The dipeptidic compounds (**4a**–**p**) were prepared by the usual Boc strategy in the liquid phase (Scheme 1). Coupling of Boc-Dmt-OH [17] with the free amino group of the P_2' ligands yielded compounds **2a**–**p**. The Boc group was deprotected with 4N HCl/dioxane, followed by coupling with Boc-Apns-OH [17] using EDC in the presence of HOBt, resulting in protected compounds **3a**–**p**. Finally, deprotection of **3a**–**p** and coupling with 2,6-dimethylphenoxyacetic acid using BOP yielded the desired dipeptide-type analogs, **4a**–**p**. Compounds **4i** and **4j** were separated by column chromatography from a *cis*-mixture, but the configurations of each diastereomer have not been established yet.

Enzyme Inhibition Assay

Recombinant HIV-1 PR was purchased from Bachem AG, Bubendorf, Switzerland. HIV PR substrate [H-Lys-Ala-Arg-Val-Tyr-Phe(*p*-NO_2)-Glu-Ala-Nle-NH_2] was synthesized by a conventional solid-phase method. The determination of HIV-1 PR inhibitory activity of the test compounds at 50 nM was based on the inhibition of cleavage of the HIV PR substrate by using recombinant HIV-1 PR (2 μg/ml) in 50 mM MES-NaOH (pH 5.5) containing 2.5 mM DTT, 1 mM EDTA-2Na, 0.2% Nonidet P-40 and 15% glycerol. After incubation for 15 min at 37°C, the reaction was terminated by the addition of 1N HCl, and the amount of *N*-terminal cleavage fragment produced was measured by HPLC.



Scheme 1 Reagents and conditions: (i) R-NH_2, TEA, BOP, DMF, rt, 18 h; (ii) anisole, 4N-HCl/dioxane, rt, 1 h; (iii) Boc-Apns-OH, HOBt, EDC, TEA, DMF, rt, 18 h; (iv) TEA, 2,6-dimethylphenoxyacetic acid, BOP, DMF, rt, 18 h.

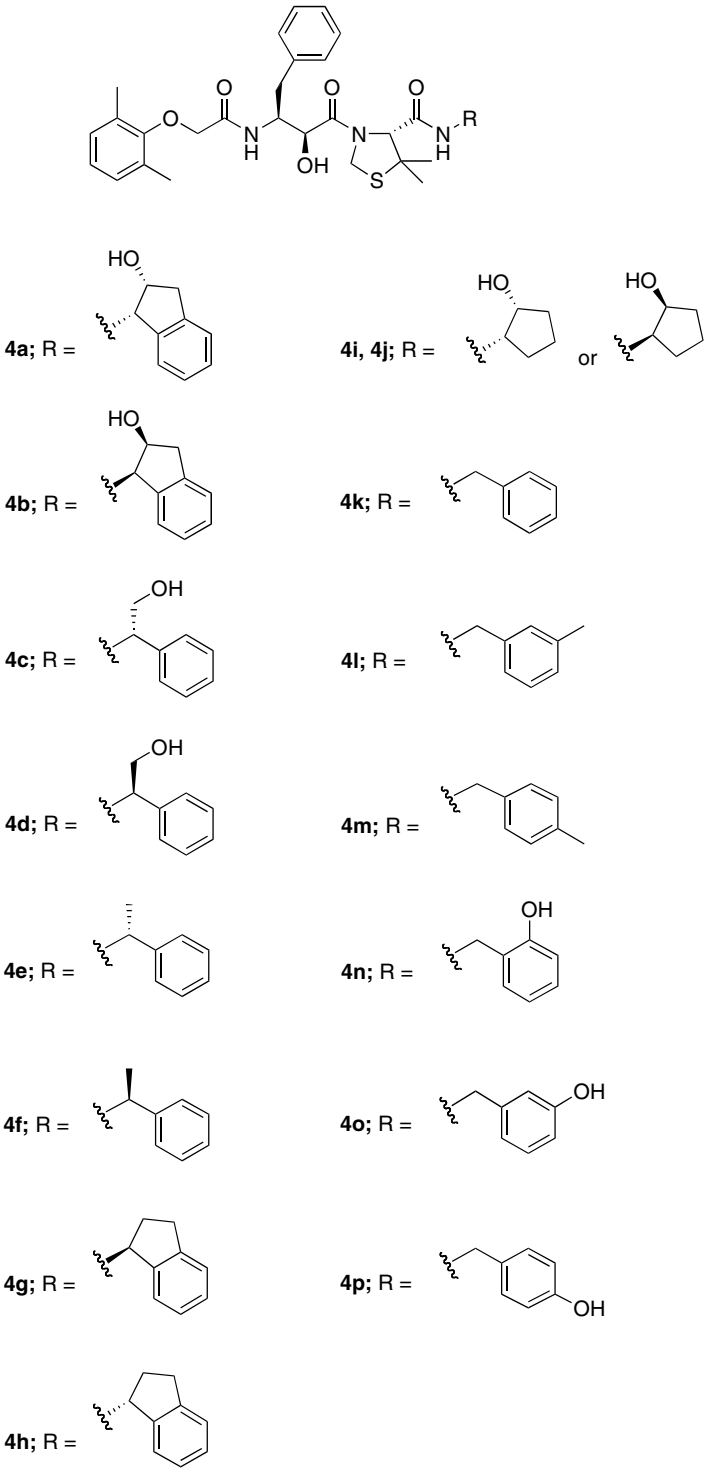


Figure 2 Structures of synthetic KNI-727 analogs.

Inhibition constants (K_i) against Plm II and Cat D were obtained as described previously [5]. Briefly, the rate of substrate hydrolysis at 25 °C was measured using 400 nM protease in 10 mM sodium formate (pH 4.0), 163 μM chromogenic substrate [H-Ala-Leu-Glu-Arg-Thr-Phe-Phe(p-NO_2)-Ser-Phe-Pro-Thr-OH] which was purchased from California Peptide Research Inc., Napa, CA and 2%

DMSO with increasing amounts of inhibitor. K_i were estimated by fitting the data to standard equations for tight binding competitive inhibitors.

RESULTS AND DISCUSSION

As shown in Table 2, the previously reported compound **4a** [6], which has (1*S*,2*R*)-1-amino-2-indanol at the P_2' position, exhibited remarkably potent inhibitory activity with a K_i value of 0.5 nM. This activity is 140-fold more potent than KNI-727. On the other hand, compound **4b**, which has the opposite configuration of **4a**, exhibited a lower inhibitory activity, suggesting that the stereochemistry at the aminoindanol part of **4a** is favorable in the interaction with the S_2' pocket of Plm II. Compounds **4c** and **4d** have a phenylglycinol with a hydroxyl group, which is more flexible than that of aminoindanol at the P_2' position, but these compounds exhibited a lower inhibitory activity than **4a**. In order to understand the significance of the hydroxyl group and the aminoindanol structure in the potent enzyme inhibition, aminoindan was introduced at the P_2' position. The inhibitory activities of the resultant compounds **4g** and **4h** were relatively low. Aminocyclopentanol was also introduced at the P_2' position, in which the phenyl ring in **4a** was removed. However, compounds **4i** and **4j** showed a lower potency. From these results, it is suggested that the tight binding observed in the aminoindanol structure at the P_2' position is due to the effect of both the hydrophobic indan structure and its spatially arranged hydroxyl group which allows for proper interaction with Plm II.

Table 2 Inhibitory Activity Against Plm II, Cat D and HIV-1 PR

	Compound	Plm II K_i (nM)	Cat D K_i (nM)	HIV-1 PR % inhibition (50 nM)
4a	(KNI-10006)	0.5	2	98
4b	(KNI-10007)	71	806	21
4c	(KNI-10043)	22	53	16
4d	(KNI-10044)	532	1040	0
4e	(KNI-10030)	11	111	41
4f	(KNI-10063)	542	nd	85
4g	(KNI-10026)	15	105	97
4h	(KNI-1269)	99	nd	71
4i	(KNI-2017a)	108	nd	89
4j	(KNI-2017b)	709	nd	41
4k	(KNI-10046)	96	260	90
4l	(KNI-10053)	41	36	58
4m	(KNI-10052)	572	1321	55
4n	(KNI-10047)	55	252	77
4o	(KNI-10048)	99	771	89
4p	(KNI-10054)	273	1642	84

nd, not determined.

The compounds (**4l**–**p**) with benzylamine derivatives exhibited lower inhibitory activities than KNI-840 which is introduced with *o*-methylbenzylamine. This result indicates that the inhibitory activity decreases in the order *o*− > *m*− > *p*-substitutions at the phenyl ring regardless of the presence of the methyl or hydroxyl group in these positions. In the case of the methyl substitution, a similar tendency was previously reported in HIV-1 PR inhibitors [17], suggesting that the recognition mechanism at the P_2' site of both Plm II and HIV-1 PR is similar.

In comparison with the Cat D inhibition, the selectivity of Plm II ($K_{i\ Cat\ D}/K_{i\ Plm\ II}$) did not improve in a series of modifications at the P_2' position (selectivity <11). Compound **4a** was also found to be a potent Cat D inhibitor ($K_i = 2$ nM). The replacement of *t*-butylamine in KNI-727 to aminoindanol in **4a** at the P_2' position contributed to a 650-fold increase in the Cat D inhibitory potency. This result suggests that a similar interaction induced by the hydroxyl group of aminoindanol enhances the binding affinity to Cat D, resulting in a decrease of the selectivity towards Plm II. This observed low selectivity, however, would not be a serious problem in future clinical use because the usual malaria treatment does not require a long time compared with the therapy of HIV infection.

As shown in Table 2, several compounds that exhibited potent Plm II inhibitory activity also exhibited potent inhibitory activities against HIV-1 PR. This is also attributed to the similarity in native substrate recognition between Plm II and HIV-1 PR. In particular, compound **4a** showed a high inhibitory activity against both Plm II and HIV-1 PR. This result suggests that the active conformations of **4a** in Plm II and HIV-1 PR may be similar. Therefore, a conformation of **4a** was obtained from the crystal structure of KNI-764 bound to HIV-1 PR (PDB entry, 2KZK) as a starting conformation. Then, its modeled structure bound to the active site of Plm II was generated adopting the conformation to the crystal structure of a complex of pepstatin A and Plm II (PDB entry, 1SME). The energy minimization with a MMFF94s force field was performed using the

Molecular Operating Environment modeling package (MOE 2003.02, Chemical Computing Group, Inc., Montreal, Canada). As shown in Figure 3, the indan moiety is fitted in the S_2' pocket of Plm II while the hydroxyl group of aminoindanol interacts with a hydroxyl group of Tyr192 by forming favorable hydrogen bonds. This suggests that the hydroxyl group of aminoindanol probably has the same role as the carbonyl group of Ser at the P_2' position in the substrate. A similar orientation of aminoindanol is shown in the case of indinavir in HIV-1 protease (PDB entry, 2BPX). However, the binding mode of aminoindanol in compound **4a** is different from the proposed model of a 1,2-dihydroxyethylene compound by Hallberg's group [12]. This is due to the fact that our modeled orientation of the phenyl group of compound **4a** is based on the active conformation of KNI-764 in HIV-1 PR.

Compound **4a** which exhibited the best activity against Plm II among all the test compounds also inhibited Plm I, IV and HAP [6]. This result suggests that compound **4a** might be able to inhibit the degradation of hemoglobin in the food vacuole. The activity of compound **4a** was also evaluated in *P. falciparum*-infected erythrocyte cultures. There was a significant reduction in the potency of compound **4a** in this assay (IC_{50} = 6.8 μM). This decrease can be explained by the fact that Plm inhibitors must penetrate four membranes (i.e. erythrocyte, parasitophorous vacuolar, parasite plasma, and food vacuole membranes) to reach their target. The excessive hydrophobicity of compound **4a** due to a dimethylphenoxyacetyl group at the P_2 position may cause a lowered cell penetration, hence suppressing its intrinsic activity.

In conclusion, novel Plm II inhibitors were designed and synthesized based on the substrate transition state. The Apns-Dmt scaffold was a Phe-Leu (P_1-P_1') transition-state mimic. Since native substrates contain a Ser residue at the P_2' position, a hydroxyl group was introduced with a fixed conformation and a bulky unit to fit the space of the S_2' pocket of Plm II. From the SAR at the P_2' position, (1*S*,2*R*)-1-amino-2-indanol was the best P_2' ligand (compound **4a**, KNI-10006) of the synthetic compounds, and both the hydroxyl group and the indan structure of the aminoindanol are important for its tight binding. Based on these observations, further modifications are under way to develop improved potential antimalarial compounds.

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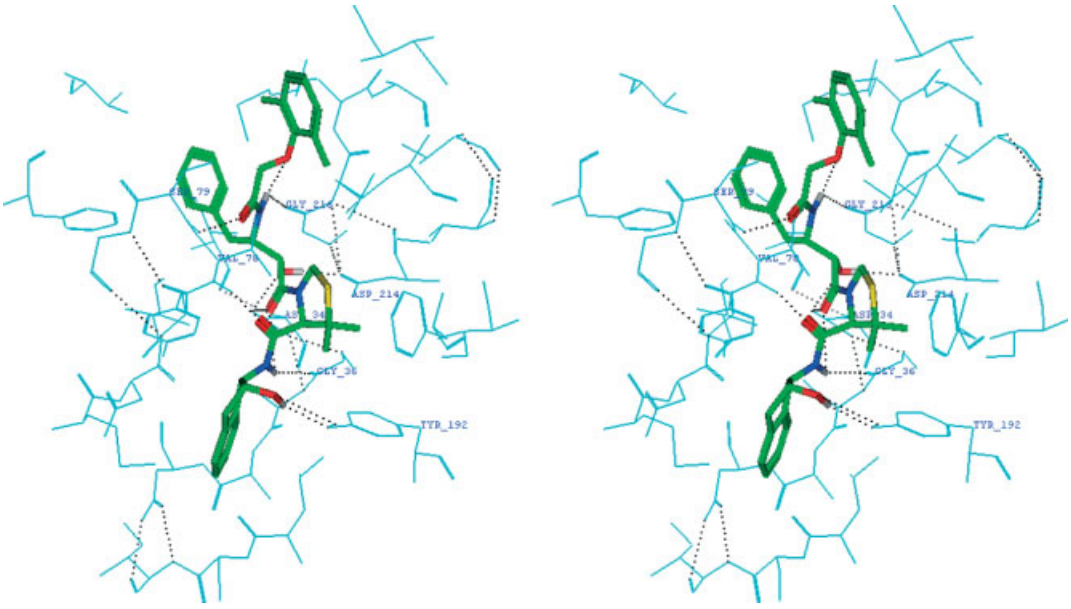

Figure 3 Modeled structure of compound **4a** bound to the active site of Plm II. Residues in the active site of Plm II are represented in light blue. Hydrogen bonds are represented with dotted line.

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