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Structural Modification of Pantothenamides Counteracts Degradation by Pantetheinase and Improves Antiplasmodial Activity

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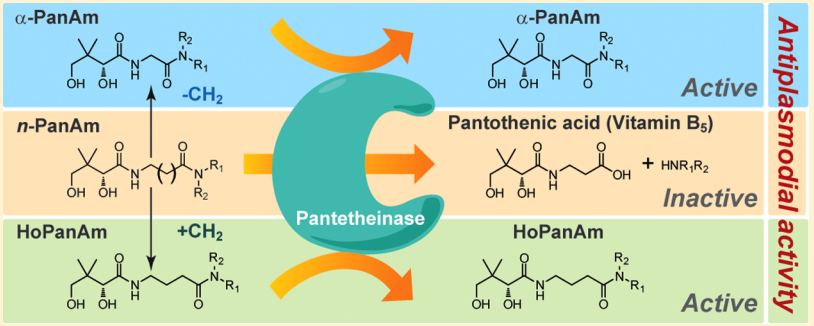
Supporting Information



ABSTRACT: Pantothenamides are secondary or tertiary amides of pantothenic acid, the vitamin precursor of the essential cofactor and universal acyl carrier coenzyme A. A recent study has demonstrated that pantothenamides inhibit the growth of blood-stage *Plasmodium falciparum* with submicromolar potency by exerting an effect on pantothenic acid utilization, but only when the pantetheinase present in the growth medium has been inactivated. Here, we demonstrate that small modifications of the pantothenamide core structure are sufficient to counteract pantetheinase-mediated degradation and that the resulting pantothenamide analogues still inhibit the in vitro proliferation of *P. falciparum* by targeting a pantothenic acid-dependent process (or processes). Finally, we investigated the toxicity of the most potent analogues to human cells and show that the selectivity ratio exceeds 100 in one case. Taken together, these results provide further support for pantothenic acid utilization being a viable target for antimalarial drug discovery.

KEYWORDS: *Pantothenamide, antimalarial, pantothenic acid, coenzyme A, pantetheinase, drug metabolism*

Nearly half the world's population is at risk of contracting malaria, a lethal infectious disease that is estimated to have caused the deaths of more than half a million people in 2010 alone.[1,2] Consequently, the search for new antimalarial agents is an ongoing pursuit that has intensified since the release of reports that *Plasmodium falciparum*, the most virulent of the protozoan parasites that cause the disease in humans, has become resistant to all chemotherapeutic agents currently in use.[3]

One set of targets that has shown promise for antimalarial drug discovery and development encompasses the processes and pathways dependent on pantothenic acid (vitamin B_5), which serves as the biosynthetic precursor to the essential metabolic cofactor and universal acyl carrier coenzyme A (CoA).[4] This is due to *P. falciparum* showing an absolute requirement for exogenous pantothenic acid,[5] and a sensitivity to compounds that interfere with its ability to utilize this vitamin. Structural analogues of pantothenic acid have shown the most promise in this regard, with many being characterized as growth inhibitors of the blood-stage parasite.[6−8] However, their exact point of action remains to be elucidated.

The pantothenamides are one class of such pantothenic acid analogues that we have recently investigated for antiplasmodial activity.[9] These compounds are prepared by transforming the carboxylic acid group of pantothenic acid to a secondary or tertiary amide and have previously been studied as antibacterial agents.[10−17] While our initial growth inhibition experiments seemed to indicate that these analogues only had modest in vitro antiplasmodial activity against blood-stage *P. falciparum*, we found this to be due to their degradation by enzyme(s) present in the human serum (or the serum substitute, Albumax II) that is used as a necessary supplement to the parasite culture medium. The Vanin proteins, which are members of the nitrilase superfamily, were identified as the probable responsible agent.[9] These proteins have pantetheinase activity, hydrolyzing

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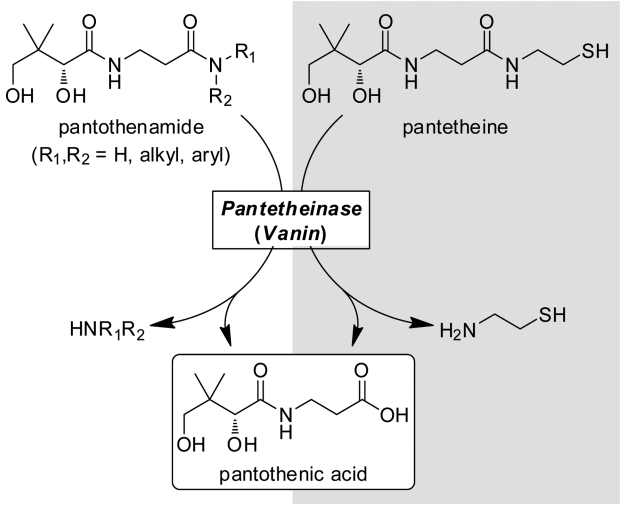
the CoA metabolite pantetheine by means of an invariant Glu−Lys−Cys catalytic triad to form pantothenic acid and the antioxidant cysteamine.[18,19] However, they tolerate a wide range of structural modifications on the cysteamine moiety, thereby explaining their ability to also degrade pantothenamides other than pantetheine (Scheme 1).[20,21]

Scheme 1. Pantothenamides and Their Degradation by the Enzyme Pantetheinase (Vanin)[a]



[a]Pantothenamides are amide analogues of pantothenic acid that are susceptible to degradation by pantetheinase. The native function of this enzyme is to hydrolyze the CoA metabolite pantetheine to pantothenic acid and cysteamine (reaction in grey box).

We found the pantothenamides' antiplasmodial potency to be significantly enhanced when growth inhibition experiments were performed using culture medium that had been preheated (or aged) to remove most of the pantetheinase activity present, with five of the compounds tested showing submicromolar IC_{50} values under these conditions. Among these, *N*-phenethyl pantothenamide displayed the highest potency with an IC_{50} of 20 ± 2 nM,[9] comparable to the IC_{50} of chloroquine when measured against parasite strains sensitive to the drug.[22] Moreover, we found the inhibitory effects of the pantothenamides to be attenuated in the presence of increased concentrations of pantothenic acid, confirming that these compounds act on pathways or processes dependent on this vitamin.

The further development of pantothenamides as antiplasmodials is therefore currently hampered by the ubiquitous presence of Vanin proteins, especially by those found in serum.[19] One approach whereby this shortcoming can be addressed is to combine pantothenamides with known pantetheinase inhibitors.[23,24] However, such a strategy would eventually require the separate optimization of the properties of two compound sets and may result in complications due to adverse interactions between them, and/or inhibition of the physiological function of pantetheinases.[19] We therefore decided to investigate an alternative approach in which the pantothenamides are structurally modified in a manner that would counteract pantetheinase-mediated degradation without compromising their antiplasmodial activity.

A previously described series of modified pantothenamides seemed ideally suited to test such an approach.[16] This series was prepared from pantothenic acid or one of two structural analogues in which its β-alanine moiety is replaced with either glycine (to give α-pantothenic acid) or γ-aminobutyric acid (to give homopantothenic acid). A range of amines representing four chemical motifs (i.e., primary alkyl amines, primary heteroatom-containing aliphatic amines, primary amines with substituents containing aromatic groups, and secondary cyclic amines) was used to introduce the amide moiety into each of these acids in parallel. In this manner, three sets of pantothenamides were formed, referred to as α-pantothenamides (α-PanAm), *n*-pantothenamides (*n*-PanAm, where *n* signifies normal), and homopantothenamides (HoPanAm), respectively (Tables 1 and S1, Supporting Information). Importantly, the structural modifications cause a displacement in the pantothenamide amide bond in the α-PanAm and HoPanAm series relative to its position in the *n*-PanAm series by either the removal or addition of a methylene group. Since previous studies showed that pantetheinase relies on the pantothenoyl moiety of its substrate for recognition,[20,21] we expected that this displacement would reduce the scissile amide bond's susceptibility to attack by the Cys residue of the enzyme's catalytic triad and thereby prevent or reduce the rate of the pantetheinase-mediated degradation of the α-PanAm and HoPanAm series relative to that of the *n*-PanAm series.

We decided to determine whether members of the α-PanAm and HoPanAm series are more resistant to pantetheinase-mediated degradation compared to their *n*-PanAm counterparts under in vitro conditions. This was tested directly by incubating four sets of pantothenamides, each with a different type of amide substituent, in the presence of recombinant human pantetheinase. The amount of amine released under these conditions was then determined periodically using a previously developed fluorescamine-based fluorescence assay (Figure 1).[9] After 24 h, the hydrolysis of the *n*-PanAm compounds was found to be complete (data not shown), in agreement with our previous findings.[9] However, under the same conditions, the hydrolysis of members of the α-PanAm and HoPanAm series was between 5 and 15% complete, except in the case of the PanAm-2 set where it approached ∼25%. This demonstrates that displacing the pantothenamide amide bond in the *n*-PanAm series confers resistance to pantetheinase degradation.

Next, we set out to determine whether the increased pantetheinase resistance of the α-PanAm and HoPanAm members (relative to the corresponding *n*-PanAm members) also translated into increased antiplasmodial potency in the presence of pantetheinase. The inhibitory activity of 47 sets of α-PanAm, *n*-PanAm, and HoPanAm compounds was determined in vitro against intraerythrocytic *P. falciparum* (strain 3D7) in 96 h growth assays initiated with parasites predominantly in the ring stage. For these tests, fresh Albumax-complete RPMI culture medium was used, to ensure the presence of pantetheinase activity.[9] From the IC_{50} values obtained in this manner (data for compounds showing IC_{50} values below 200 μM are given in Table 1; the complete data set is given in Table S1, Supporting Information), it is clear that displacement of the scissile amide bond in a *n*-PanAm significantly improves its antiplasmodial activity ($P < 0.05$ and $P < 0.01$ for α-PanAm and HoPanAm members, respectively, compared to *n*-PanAm members; unpaired *t* test) as predicted. To confirm that the increased potency of the α-PanAm and HoPanAm members is due to their resistance to pantetheinase degradation, the antiplasmodial activity of 11 selected sets of α-PanAm, *n*-PanAm, and HoPanAm

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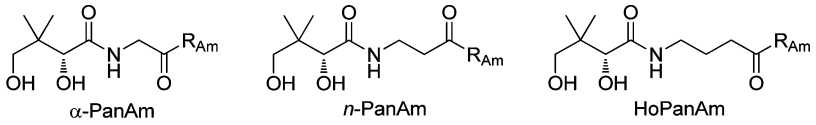
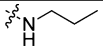
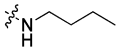
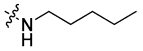
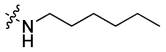
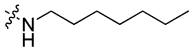
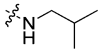
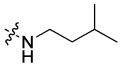
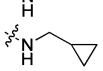
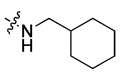
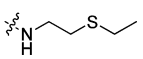
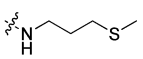
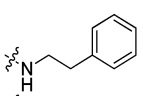
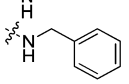
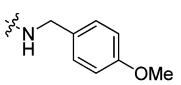
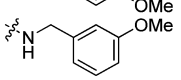
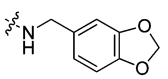
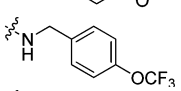
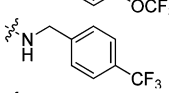
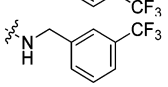
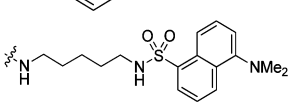
Table 1. Inhibitory Activity of Selected Pantothenamides on the in Vitro Proliferation of *P. falciparum*



PanAm Entry	R_{Am}	IC_{50} (μM) in fresh medium[a]			IC_{50} (μM) in aged medium[a]		
		α-PanAm	*n*-PanAm	HoPanAm	α-PanAm	*n*-PanAm	HoPanAm
1		13 ± 1	199 ± 53	13 ± 1	ND[b]	ND	ND
2		5.3 ± 2.1	>200	11 ± 1	ND	ND	ND
3		7.0 ± 1.4	182 ± 17	3.9 ± 0.4	14 ± 2	7.5 ± 6.2	2.2 ± 0.1
4		4.7 ± 0.1	134 ± 6	8.0 ± 0.7	7.9 ± 1.8	0.55 ± 0.01	14 ± 7
5		46 ± 3	>200	11 ± 1	ND	ND	ND
9		18 ± 2	200 ± 1	11 ± 2	ND	1.1 ± 0.1[c]	ND
11		12 ± 3	>200	8.9 ± 0.3	ND	ND	ND
14		6.4 ± 0.7	149 ± 7	12 ± 1	ND	ND	ND
15		164 ± 2	>200	2.1 ± 0.2	140 ± 40	>200	3.3 ± 0.5
18		10 ± 1	87 ± 13	2.0 ± 0.1	11 ± 1	0.28 ± 0.02	1.9 ± 0.1
20		38 ± 7	143 ± 5	7.7 ± 0.1	ND	0.23 ± 0.06[c]	ND
29		3.4 ± 0.8	53 ± 11	2.1 ± 0.1	ND	0.020 ± 0.002[c]	ND
30		122 ± 28	>200	6.1 ± 0.5	127 ± 16	>200	4.7 ± 0.5
31		26 ± 6	>200	9.5 ± 0.7	71 ± 8	>200	10 ± 1
32		>200	>200	21 ± 1	>200	>200	22 ± 1
34		53 ± 17	144 ± 13	2.4 ± 0.4	ND	ND	ND
35		71 ± 9	>200	6.3 ± 1.1	64 ± 16	136 ± 34	4.9 ± 1.3
36		48 ± 8	107 ± 2	3.5 ± 0.2	45 ± 6	117 ± 12	2.0 ± 0.1
37		89 ± 10	>200	4.0 ± 0.6	116 ± 21	156 ± 22	1.9 ± 1.0
38		155 ± 27	>200	1.1 ± 0.1	ND	ND	ND

[a]Inhibition of the proliferation of *P. falciparum* cultured (for 96 h) in either fresh (i.e., freshly prepared) or aged (i.e., heat-treated to reduce pantetheinase activity) Albumax-complete RPMI containing 1 μM pantothenic acid (see Supporting Information for details). Values represent the mean ± SEM (or range/2) from at least two independent experiments, each performed in triplicate. [b]ND, not determined. [c]Values taken from ref 9.

pantothenamides was tested in aged medium (i.e., medium subjected to prolonged heat-treatment to reduce the pantetheinase activity). The results show that, while the potency of the *n*-PanAm members improve under these conditions (consistent with our previous results[9]), the activity of the α-PanAm and HoPanAm members determined in the

dx.doi.org/10.1021/ml400180d | ACS Med. Chem. Lett. 2013, 4, 784−789

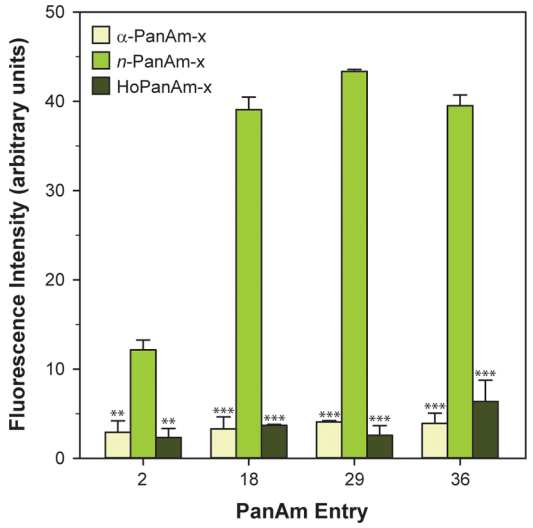


Figure 1. Pantetheinase-mediated hydrolysis of the α-PanAm, *n*-PanAm, and HoPanAm series of pantothenamides. Four sets of α-PanAm, *n*-PanAm, and HoPanAm compounds (see Table 1 for structures) were treated with recombinant human pantetheinase. After 24 h, the amount of amine released was determined by means of a fluorescamine-based fluorescence assay. Values represent the mean from three independent experiments, each performed in duplicate; the error bars represent SEM. Asterisks above the bars indicate the significance of the difference between the values determined for the respective α-PanAm and HoPanAm series members and the *n*-PanAm in a given set (** $P < 0.005$; *** $P < 0.001$, one way ANOVA). Note that, since the fluorescence intensity of fluorescamine–amine conjugates is dependent on the structure of the amine, the fluorescence measurements are not necessarily comparable between the different sets.

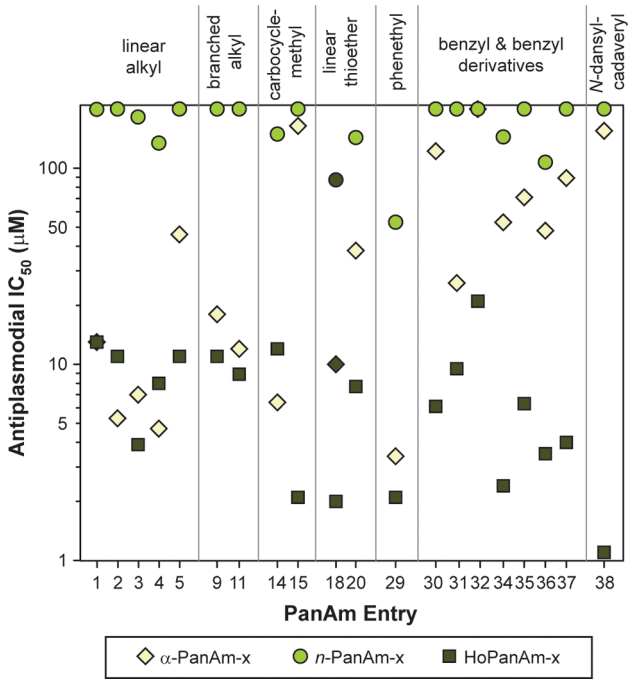


Figure 2. Structure–activity relationship analysis of the inhibitory activity of selected pantothenamides on the in vitro proliferation of *P. falciparum* determined in fresh medium. Graphical representation of the data given in Table 1, highlighting the differences between the activities of the α-PanAm, *n*-PanAm, and HoPanAm members, and the influence of the structure of the amide substituent (structural group descriptions are given above the graph) on potency. Symbols touching the top of the graph represent compounds with IC_{50} values > 200 μM.

presence and absence of pantetheinase is comparable ($P > 0.05$; unpaired *t* test). Taken together, these results confirm that the structural modification of pantothenamides is a viable approach to confer resistance to pantetheinase degradation while at the same time preserving antiplasmodial activity.

A comparison of the IC_{50} values determined for the various pantothenamides in fresh medium reveals several interesting trends (Figure 2). First, while the α-PanAm members show variable antiplasmodial potencies, the majority of inhibitory HoPanAm members have IC_{50} values below 10 μM, with the most potent compound (HoPanAm-38) having an IC_{50} value of 1.1 ± 0.1 (mean ± SEM; $n = 4$). This highlights the elongation of the β-alanine moiety of the *n*-PanAm series as the preferred structural modification to counteract pantetheinase degradation while maintaining antiplasmodial activity. Second, there does not seem to be a clear structure–activity relationship between the α-PanAm, *n*-PanAm, and HoPanAm series from the perspective of an analysis that focuses on the amide substituent. This would not be unexpected if all three series interacted with the same target since the shortening or elongation of the β-alanine moiety by one methylene unit would displace the amide substituent relative to the pantoyl moiety that all three hold in common. Therefore, one might predict that compounds that have a similar but opposite modification in the amide substituent would counteract these effects and should show some correlation in potency. A case in point is the phenethyl-substituted *n*-PanAm-29 that shows potent (20 ± 2 nM; mean ± SEM; $n = 3$)[9] activity in aged medium. Displacement of the phenyl group of *n*-PanAm-29 (as in the case of *n*-PanAm-30) leads to a complete loss of activity (IC_{50} > 200 μM); in contrast, the corresponding HoPanAm-30, which has an extension that returns the phenyl group to the same relative position as in *n*-PanAm-29, has an IC_{50} value of 4.7 ± 0.5 μM (mean ± range/2; $n = 2$). Similar examples can be found among the alkyl-substituted pantothenamides (Table 1, entries 3 and 4), suggesting that all three series of pantothenamides interact with the same target(s).

We next set out to confirm that the α-PanAm and HoPanAm members are on-target and, like *n*-PanAm members, affect processes in *P. falciparum* dependent on pantothenic acid.[9] This was done by investigating the antimalarial activity of these compounds in the presence of excess pantothenic acid (100 μM, compared to the 1 μM that is normally present in culture medium). Concentration–response assays were conducted in fresh culture medium with 25 compounds selected on the basis of their antiplasmodial activity and structural diversity (Table 2). A significant increase ($P < 0.01$, unpaired *t* test) in IC_{50} values was observed for nearly all the compounds tested, with potency reduced by >95-fold in one case. The only exceptions were three α-PanAm members with aromatic substituents (α-PanAm-36, -37, and -38) that showed significant but small ($P \approx 0.01$) or negligible shifts ($P > 0.1$). While the exact basis for this difference is not clear, for the other α-PanAm and HoPanAm members tested these results indicate that these compounds, like the *n*-PanAm pantothenamides, exert their inhibitory effects by affecting targets dependent on pantothenic acid. This conclusion is strengthened further by the fact that the HoPanAm members, which in general showed the highest potency in fresh medium (Table 1), also showed the largest fold shifts when the concentration of pantothenic acid was

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Table 2. Effect of Pantothenic Acid Supplementation on Pantothenamide Potency in Fresh Medium[a]

	IC_{50} (μM) in fresh medium with added pantothenic acid[a]		fold shift	
PanAm entry	α-PanAm	HoPanAm	α-PanAm	HoPanAm
3	142 ± 16	195 ± 5	20	50
4	104 ± 4	199 ± 6	22	25
14	183 ± 8	ND[b]	29	ND
15	ND	>200	ND	>95
18	200 ± 19	169 ± 8	19	85
29	135 ± 6	139 ± 10	40	68
30	>200	>200	>2	>33
31	189 ± 12	>200	7	>21
32	ND	>200	ND	>10
34	>200	88 ± 24	>4	37
35	>200	>200	>3	>32
36	57 ± 4	59 ± 14	1	17
37	177 ± 8	169 ± 24	2	42
38	157 ± 19	38 ± 1	1	35

[a]Inhibition of proliferation of *P. falciparum* cultured (for 96 h) in fresh (i.e., in the presence of active pantetheinase) Albumax-complete RPMI containing 100 μM pantothenic acid (see Supporting Information for details). Fold shift gives the ratio of the IC_{50} values determined in fresh medium (Table 1) to those determined in the presence of added pantothenic acid. Values represent the mean ± range/2 from two independent experiments, each performed in triplicate. [b]ND, not determined.

increased. A discussion on the possible identity of these targets is provided in the Supporting Information.

We finally also explored the potential utility of the 10 most potent α-PanAm and HoPanAm members identified in this study as antimalarial drugs by investigating their toxicity to mammalian cells. This was done by determining their concentration−response profiles against Jurkat cells (a human leukemic T-cell line). The toxicity results (Table 3) show that although no obvious trend was apparent for these compounds, the selectivity indexes (the ratio of the IC_{50} values measured against Jurkat cells to the IC_{50} values for the inhibition of *P. falciparum* growth) of HoPanAm-3, -15, -18, -29, and -37 ranged from 40 to >100, indicating that at least these pantothenamides should be considered for in vivo antimalarial activity studies in a mouse model of malaria.

Table 3. Toxicity Assessment of the Most Potent α-PanAm and HoPanAm Series Members

PanAm entry	IC_{50} (μM) vs Jurkat cells[a]	selectivity index
α-PanAm-4	126 ± 13	27
α-PanAm-29	53 ± 5	16
HoPanAm-3	192 ± 5	49
HoPanAm-15	113 ± 6	54
HoPanAm-18	>200	>100
HoPanAm-29	99 ± 16	47
HoPanAm-34	23 ± 4	9
HoPanAm-36	13 ± 1	4
HoPanAm-37	161 ± 23	40
HoPanAm-38	31 ± 12	28

[a]Jurkat cells were cultured for 96 h in medium containing 1 μM pantothenic acid. Values represent means ± SEM from three independent experiments performed in triplicate. The selectivity index for each compound indicates the ratio of the IC_{50} values obtained for the inhibition of Jurkat cell growth and in vitro parasite proliferation in fresh medium.

In conclusion, we have demonstrated in this study that antiplasmodial pantothenamides can be structurally modified to counteract their susceptibility to degradation by pantetheinase while at the same time maintaining their on-target antiplasmodial activity. The good selectivity for parasite growth inhibition (compared to human cell inhibition) of the most potent pantetheinase-resistant pantothenamides identified lends further support for continued focus on these antivitamins as potential new antimalarials.

■ ASSOCIATED CONTENT

Supporting Information

Full set of inhibition results (Table S1), supplementary discussion, and all experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.d.V. and C.M. performed the experiments and data analysis. Y.H. provided initial data that contributed to the conclusions. M.d.V., C.M., C.S., E.S., and K.J.S. contributed to experimental design and data interpretation. M.d.V., E.S., and K.J.S. wrote the paper with input from all the authors. All authors have approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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