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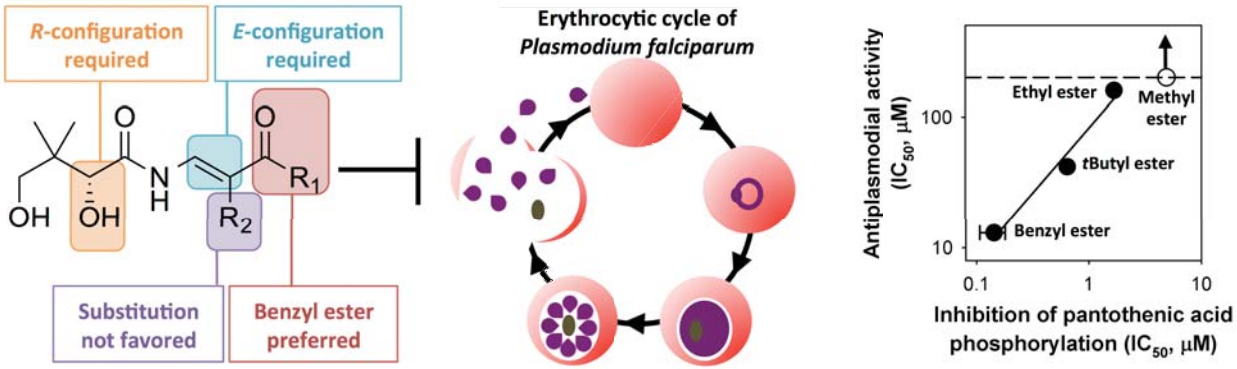
R-configuration required
E-configuration required
O
O
N
H
OH
OH
R2
R1
Substitution not favored
Benzyl ester preferred
Erythrocytic cycle of
Plasmodium falciparum
Antiplasmodial activity
(IC50, µM)
100
10
Ethyl ester
Methyl ester
tButyl ester
Benzyl ester
0.1
1
10
Inhibition of pantothenic acid
phosphorylation (IC50, µM)

Structure-activity analysis of CJ-15,801 analogues that interact with *Plasmodium falciparum* pantothenate kinase and inhibit parasite proliferation

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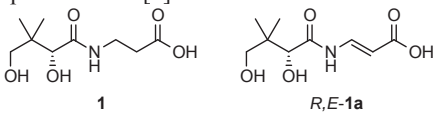
ABSTRACT

Survival of the human malaria parasite *Plasmodium falciparum* is dependent on pantothenate (vitamin B_5), a precursor of the fundamental enzyme cofactor coenzyme A. CJ-15,801, an enamide analogue of pantothenate isolated from the fungus *Seimatosporium* sp. CL28611, was previously shown to inhibit *P. falciparum* proliferation *in vitro* by targeting pantothenate utilization. To inform the design of next generation analogues, we set out to synthesize and test a series of synthetic enamide-bearing pantothenate analogues. We demonstrate that conservation of the *R*-pantoyl moiety and the *trans*-substituted double bond of CJ-15,801 is important for the selective, on-target antiplasmodial effect, while replacement of the carboxyl group is permitted, and, in one case, favored. Additionally, we show that the antiplasmodial potency of CJ-15,801 analogues that retain the *R*-pantoyl and *trans*-substituted enamide moieties correlates with inhibition of *P. falciparum* pantothenate kinase (*Pf*PanK)-catalyzed pantothenate phosphorylation, implicating the interaction with *Pf*PanK as a key determinant of antiplasmodial activity.

1. Introduction

Malaria is a widespread and lethal infectious disease transmitted by the female *Anopheles* mosquito and caused by parasites of the genus *Plasmodium*. There are six species of *Plasmodium* responsible for infections in humans [1,2]. Of these, *P. falciparum* is the most deadly [3] due to its ability to evade host defences during the blood stage of its development. Worldwide, there were an estimated 214 million new cases of malaria, with 438,000 deaths, in 2015 [3]. Children under the age of five years accounted for 306,000 of the deaths [3].

P. falciparum is reliant on exogenous pantothenic acid (**1**, Figure 1; also referred to as vitamin B_5, and pantothenate, when ionized) a precursor of the ubiquitous enzyme cofactor coenzyme A (CoA). Analogues of pantothenate have been shown to compromise the parasite's ability to utilize this vitamin and thereby inhibit parasite proliferation [4-11], making them promising leads for new antimalarials. CJ-15,801 (*R,E*-**1a**), an analogue of pantothenate containing a *trans* enamide unit, was isolated from the fermentation broth of the fungus *Seimatosporium* sp. CL28611 [12]. Following on from the initial observation that CJ-15,801 inhibits proliferation of *Staphyloccocus aureus* strains [12], CJ-15,801 was tested against blood stage *P. falciparum* parasites and shown to inhibit proliferation *in vitro*, with an IC_{50} value (the concentration yielding 50% inhibition) of 39 μM [9]. Importantly, the antiplasmodial effect is alleviated by increasing the concentration of pantothenate in the growth medium, consistent with the compound exerting an effect on parasite proliferation specifically by inhibiting pantothenate utilization or a process dependent on pantothenate [9].



1 *R,E*-**1a**

Figure 1. Pantothenic acid (**1**) and CJ-15,801 (*R,E*-**1a**).

Owing to both the biological activity and interesting structure of CJ-15,801, this natural product has attracted significant interest. Correspondingly, a number of different approaches to the synthesis of CJ-15,801, with differing degrees of stereoselectivity, have been described [13-17].

Recent mechanistic investigations have revealed that in *S. aureus* CJ-15,801 is phosphorylated by pantothenate kinase (PanK), the enzyme that catalyzes the phosphorylation of pantothenate in the first step of its transformation into CoA [18]. Subsequently, phospho-CJ-15,801 is accepted as a substrate by the next enzyme in the pathway (phosphopantothenoylcysteine synthetase, PPCS), and reacts to become cytidylylated. The cytidylylated phospho-CJ-15,801, which closely mimics the natural reaction intermediate and binds tightly and reversibly to the enzyme, does not react further and instead inhibits the enzyme [18]. Whether a similar mechanism of action operates in *P. falciparum* remains to be determined.

For the purpose of exploring the structural requirements for on-target antiplasmodial activity and probing the antiplasmodial mechanism of action of CJ-15,801, we synthesized a series of novel enamide-bearing pantothenic acid analogues. Herein, we describe the synthesis of the new analogues, report on their effect on proliferation of *P. falciparum* and human cells, and in a *Pf*PanK-catalyzed pantothenate phosphorylation assay. The study yielded a selective inhibitor of *P. falciparum* proliferation with improved activity relative to CJ-15,801, and structure-activity relationship (SAR) information that can be used to inform design of a new generation of more potent antiplasmodial pantothenate analogues. We also provide data consistent with *Pf*PanK playing a role in the mechanism of action of these compounds.

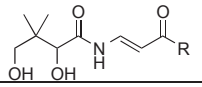
2. Results and discussion

2.1 Chemistry

To enable an exploration of the effect of double bond geometry, configuration about the chiral center, and carboxylate replacement on the antiplasmodial activity of CJ-15,801, compounds **1a-e** (Table 1) were synthesized. The effect of double bond substitution, and the importance of the "pantoyl" (2,4-dihydroxy-3,3-dimethylbutyryl) moiety, were also explored (alone and in combination) through testing of enamides **1-2c$_{Me}$** and **2-7c** (Table 2).

The synthesis of all enamides was achieved in high yield through olefination of the *N*-formyl imide precursors (prepared from the corresponding amides) using previously described methods (**Schemes 1 – 3**) [16,19]. The *E/Z* isomers were separated by flash column chromatography. CJ-15,801 and enamide analogues **1a-e** and **1c$_{Me}$** (all of which possess a diol moiety) were synthesized from carboxamide **2g** (**Schemes**

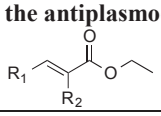
Table 1. Antiplasmodial activity of CJ-15,801 analogues.



Compound	R	IC_{50} value against *P. falciparum* (μM)[a]			
		R		*S*	
		trans	*cis*	*trans*	*cis*
1a	OH	36 ± 4 (> 2.8)[b]	> 200	> 100	> 100
1b	OMe	> 200	> 200	> 100	> 100
1c	OEt	159 ± 12 (> 1.3)	> 200	> 100	> 100
1d	OBn	13 ± 1 (> 7.7)	89 ± 11 (0.9 ± 0.1)	> 100	> 100
1e	O*t*-Bu	42 ± 5 (3.1 ± 0.4)	39 ± 2 (1.0 ± 0.1)	> 100	> 100

[a]IC_{50} values measured against *P. falciparum* parasites cultured (for 96 h) in medium containing 1 μM pantothenate are shown. The data are averages from 2 – 7 independent experiments, each performed in triplicate. IC_{50} values below the highest concentration tested are presented as the mean ± range/2 (n = 2) or SEM (n = 3 – 7). [b]Values in brackets show the fold-increase in IC_{50} value measured upon raising the pantothenate concentration from 1 to 100 μM. The fold-increase was calculated by dividing the IC_{50} value measured against *P. falciparum* parasites cultured (for 96 h) in medium containing 100 μM pantothenate by that measured in a paired experiment against parasites cultured in medium containing 1 μM pantothenate. The data are averages from two independent experiments, each performed in triplicate and errors represent range/2.

Table 2. Effect of double bond substitution and pantoyl replacement on the antiplasmodial activity of enamides.



Compound	R_1	R_2	IC_{50} value against *P. falciparum* (μM)[a] (1 μM pantothenate)	Fold increase in IC_{50} with 100-fold increase in pantothenate[b]
R,E-**1c**		H	159 ± 12	> 1.3
R,E-**1c$_{Me}$**		Me	> 200	ND
R,E-**2c**		H	144 ± 9	0.9 ± 0.1
R,E-**2c$_{Me}$**		Me	93 ± 18	1.0 ± 0.1
E-**3c**		H	158 ± 25	± 0.1
E-**3c$_{Me}$**		Me	120 ± 18	1.0 ± 0.1
E-**4c**		H	> 200	ND
E-**5c**		H	181 ± 15	0.9 ± 0.1
E-**6c**		H	93 ± 5	1.0 ± 0.1
E-**7c**		H	61 ± 6	1.4 ± 0.1

[a]The IC_{50} values measured against *P. falciparum* parasites cultured (for 96 h) in medium containing 1 μM pantothenate, are shown. The data are averages from 2 – 4 independent experiments each performed in triplicate. IC_{50} values below the highest concentration tested are presented as the mean ± SEM (n = 3 or 4). [b]In brackets is shown the fold-increase in IC_{50} value measured upon raising the pantothenate concentration from 1 to 100 μM. The fold-increase was calculated by dividing the IC_{50} value measured against *P. falciparum* parasites cultured (for 96 h) in medium containing 100 μM pantothenate by that measured in a paired experiment against parasites cultured in medium containing 1 μM pantothenate. The data are averages from two independent experiments, each performed in triplicate and errors represent range/2.

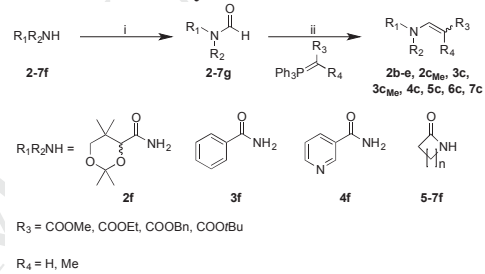
2 and **3**). Treatment of the acetonide-bearing enamides with $BiCl_3$ under previously described conditions [16] afforded the desired free diols (**Scheme 2**). CJ-15,801 and its stereoisomers (all with carboxylic acid units) were synthesized via the trimethylsilyl (TMS) esters **2h** (**Scheme 3**), a strategy that allowed the orthogonal deprotection of the carboxylic acid and diol units chemoselectively [16].

2.2 Antiplasmodial activity

The effect of the enamide analogues of pantothenate on proliferation of intraerythrocytic stage *P. falciparum in vitro* was assessed in 96 h proliferation assays. An IC_{50} value of 36 ± 4 μM (mean ± SEM, n = 3) was determined for the chemically synthesized CJ-15,801 (Table 1), which matched that determined for CJ-15,801 isolated from the fungus *Seimatosporium* (39 ± 3 μM) [9]. Methyl ester analogue *R,E*-**1b**, which was previously shown to lack inhibitory activity against *S. aureus* [18], was also observed to be devoid of antiplasmodial activity. By contrast, however, ester analogues *R,E*-**1c-e** were found to inhibit proliferation of *P. falciparum*, consistent with the free carboxylic acid not being required for activity. The highest activity was observed for the benzyl ester analogue (*R,E*-**1d**), which was approximately threefold more potent than CJ-15,801 (IC_{50} = 13 ± 1 μM; mean ± SEM, n = 6). *cis*-CJ-15,801 (*R,Z*-**1a**) and its ester analogues *R,Z*-**1c-d** were less active than their *trans* counterparts.

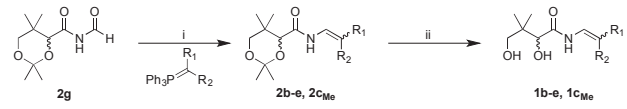
None of the analogues with *S*-configuration at the chiral center of the pantoyl moiety (the opposite configuration of CJ-15,801) were found to inhibit parasite proliferation with IC_{50} values below 100 μM, revealing a requirement for the natural *R*-configuration. For one ester analogue of CJ-15,801 (ethyl ester *R,E*-**1c**), the effect of substitution of the double bond and replacement of the pantoyl moiety was investigated (Table 2). While introduction of a methyl substituent and replacement of the pantoyl moiety with a pyridine ring were not tolerated ($IC_{50} \geq$ 200 μM), replacement of the pantoyl moiety with a ketal group, phenyl ring, or incorporation of the amide group into a lactam ring, was, and in a few cases, an increase in antiplasmodial activity was observed (e.g. lactam *E*-**7c**).

Scheme 1. General synthesis of enamides from amides.



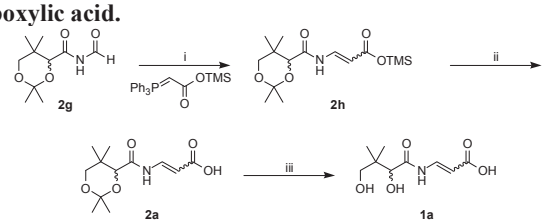
Reagents and conditions: (i) *n*-BuLi, THF, 0 °C, 0.5 h then *N*-formylbenzotriazole, THF, 0 ºC, 50 – 81%; (ii) C_6H_6, 80 °C, 18 h, 37 – 98%.

Scheme 2. Synthesis of CJ-15,801 analogues 1b-e and 1c$_{Me}$.



Reagents and conditions: (i) C_6H_6, 80 °C, 18 h, 87 – 98%; (ii) $BiCl_3$, aq. MeCN, RT, 3 – 18 h, 29 – 71%.

Scheme 3. Synthesis of CJ-15,801 and isomers with a carboxylic acid.



Reagents and conditions: (i) C_6H_6, 80 °C, 18 h, 96%; (ii) TBAF (1M in THF), THF, 0 °C, 20 h, 84% (*trans*), 92% (*cis*); (iii) $BiCl_3$, aq. MeCN, RT, 3 – 18 h, 63% (*trans*), 26% (*cis*).

When the concentration of pantothenate in the growth medium was increased 100-fold, the effect of CJ-15,801 was alleviated (Figure 2A). This result, which parallels a previous observation made with CJ-15,801 isolated from the fungus

that produces it [9], is consistent with the compound exerting an antiplasmodial effect through inhibition of CoA biosynthesis and/or utilization. We therefore set out to investigate the effect of increasing the pantothenate concentration on the antiplasmodial activity of the additional enamide analogues for which IC_{50} values had been determined.

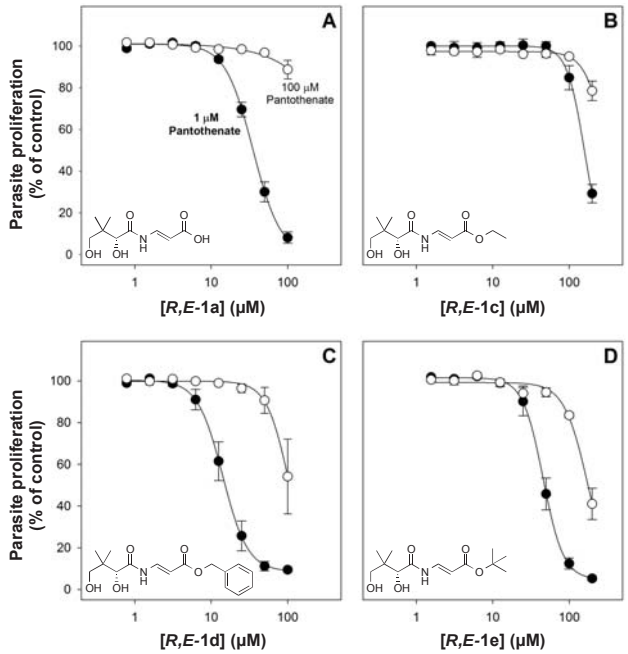


Figure 2. Effect of pantothenate supplementation on the antiplasmodial activity of CJ-15,801 (**A**) and enamides *R,E*-**1c** (**B**), *R,E*-**1d** (**C**) and *R,E*-**1e** (**D**). The effect of increasing concentrations of the enamides on proliferation of *P. falciparum* parasites cultured (for 96 h) in medium containing 1 µM (closed circles) or 100 µM (open circles) pantothenate is shown. The data obtained with parasites cultured in 1 µM pantothenate are from 3 – 8 independent experiments each performed in triplicate. The data obtained with parasites cultured in the presence of 100 µM pantothenate are from 2 – 4 independent experiments each performed in triplicate. Error bars represent range/2 or SEM, and where not shown, are smaller than the symbol.

As shown in Figure 2B-D, increasing the pantothenate concentration 100-fold resulted in attenuation of the antiplasmodial activity of compounds *R,E*-**1c-e** – all enamides with both a pantoyl moiety with *R*-configuration and a *trans*-substituted double bond (analogous to CJ-15,801). This is consistent with these compounds also acting through inhibition of CoA biosynthesis and/or utilization. The attenuating effect of pantothenate was greatest for the more active of these compounds (CJ-15,801, *R,E*-**1d** and *R,E*-**1e**). This is likely to be because the lower concentrations of these analogues required for an on-target antiplasmodial effect are farther from concentrations with a non-specific toxic effect, as compared with the less active *R,E*-**1c**. Interestingly, increasing the pantothenate concentration 100-fold does not attenuate the activity of *R,Z*-**1d-e** (the active *R*-pantoyl enamides with a *cis*-substituted double bond; Table 1), consistent with these compounds, by contrast, acting via a different mechanism. Similarly, increasing the pantothenate concentration has no effect on the activity of the active enamides without the pantoyl moiety (Table 2), except for *E*-**7c**, for which a small (1.4-fold) increase in IC_{50} was observed. This finding implicates both the *R*-pantoyl moiety and *trans*-substituted double bond as being required for a CoA biosynthesis/utilization-related antiplasmodial effect.

2.3 Inhibition of pantothenate phosphorylation

Previously it was shown that CJ-15,801 inhibits the accumulation of pantothenate (a combination of transport and subsequent trapping by phosphorylation) within *P. falciparum* parasites [9]. Here we investigated directly whether this was a consequence of inhibition of pantothenate phosphorylation by measuring phosphorylation of pantothenate by PanK in *P. falciparum* lysate in the presence of CJ-15,801. When tested at 100 µM, CJ-15,801 was observed to inhibit pantothenate phosphorylation by 87 ± 1% (mean ± SEM, n = 3, Figure 3). Of the additional enamides with a free diol moiety (**1a-e** and **1c$_{Me}$**), all those with both *R*-configuration and a *trans*-substituted double bond also inhibited pantothenate phosphorylation (including those without an effect on parasite proliferation at the same concentration). Ester analogues *R,E*-**1b-e** showed the greatest effect, inhibiting phosphorylation of pantothenate by > 90% at 100 µM. The effect of the same concentration of the corresponding enamides with a free diol moiety with *S*-configuration and/or a *cis*-substituted double bond (*R,Z*-, *S,E*-, or *S,Z*-**1a-e**) was notably less (Figure 3); with a few exceptions (*S,E*-**1b**, *R,Z*-**1c**, and *R,Z*-, *S,E*-, and *S,Z*-**1d**) < 25% inhibition was observed. The results suggest that although some enamides with a *S*-pantoyl moiety and/or a *cis*-substituted double bond are able to interact with *Pf*PanK, *R*-configuration and a *trans*-substituted enamide moiety is required for more potent inhibition of pantothenate phosphorylation. The enamides that do not retain the free diol moiety generally had little-to- no-effect on pantothenate phosphorylation (Supporting information Figure S1).

IC_{50} values for inhibition of pantothenate phosphorylation were determined for the enamides with a free pantoyl moiety with *R*-configuration and a *trans*-substituted double bond

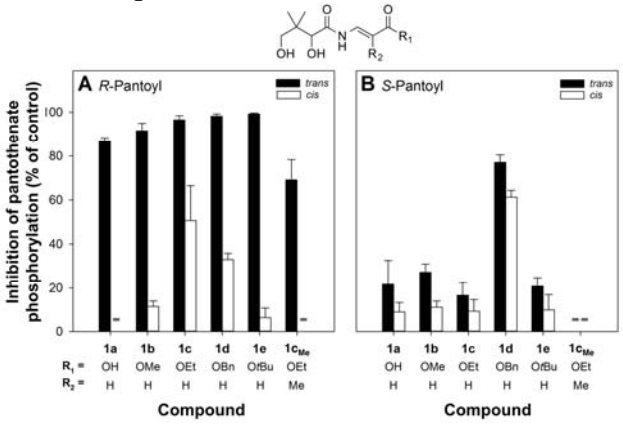


Figure 3. Effect of enamide analogues of pantothenate on *Pf*PanK-catalyzed pantothenate phosphorylation. [^{14}C]Pantothenate phosphorylation by PanK in *P. falciparum* lysate was measured in the presence of 100 µM of each enamide. The concentration of pantothenate present in the assay was 1.8 µM (*R*-compounds, **A**) or 1 µM (*S*-compounds, **B**). The percentage inhibition was calculated from the measured amounts of [^{14}C]pantothenate phosphorylated during a pre-determined incubation period (in which phosphorylation increased linearly with time in the absence of inhibitors) in the presence of enamide or the corresponding concentration of DMSO only. The data are from 3 – 8 independent experiments, each performed in duplicate. Error bars represent SEM. A dash in place of a column indicates that the compound was not tested in the assay.

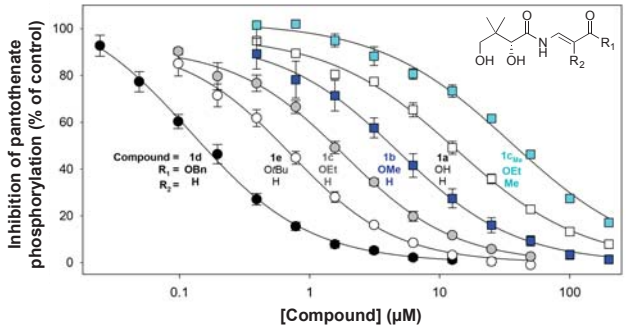


Figure 4. Concentration-dependent effect of enamides with a *R*-pantoyl moiety and *trans*-substituted double bond on *Pf*PanK-catalyzed pantothenate phosphorylation. [^{14}C]Pantothenate phosphorylation by PanK in *P. falciparum* lysate was measured in the presence of 1.8 μM pantothenate. The percentage inhibition was calculated from the measured amounts of [^{14}C]pantothenate phosphorylated during a 15 min incubation (in which phosphorylation increased linearly with time in the absence of inhibitors) in the presence or absence of increasing concentrations of each enamide. The data are from 2 – 5 independent experiments, each performed in duplicate. Error bars represent range/2 or SEM, and where not shown, are smaller than the symbol.

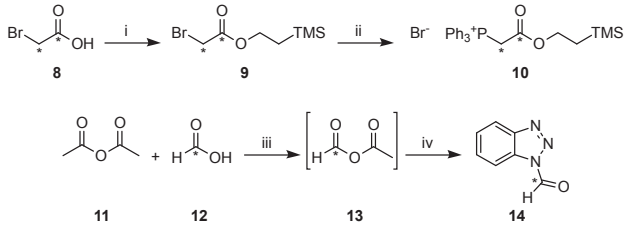
(Figure 4). The ester analogues of CJ-15,801 (*R,E*-**1b-e**) were all found to inhibit pantothenate phosphorylation more effectively than CJ-15,801, with the most effective inhibitor – benzyl ester *R,E*-**1d** – inhibiting pantothenate phosphorylation with an IC_{50} value of 140 ± 40 nM (mean ± SEM, n = 5), 100-fold lower than that of CJ-15,801 (IC_{50} = 14 ± 3 μM, mean ± SEM, n = 3; Figure 4). Introduction of a methyl substituent to the double bond (as in compound **1c$_{Me}$**) was observed to reduce inhibitory activity. Notably, for these ester analogues of CJ-15,801, the same trend was observed for inhibitory activity against pantothenate phosphorylation as for antiplasmodial activity (benzyl ester *R,E*-**1d** > *tert*-butyl ester *R,E*-**1e** > ethyl ester *R,E*-**1c** > methyl ester *R,E*-**1b**, and ethyl ester with methyl substituent *R,E*-**1c$_{Me}$**). CJ-15,801, however, notably does not fit this trend – showing only a modest effect on pantothenate phosphorylation despite being among the best inhibitors of parasite proliferation.

The enamide analogues of pantothenate may inhibit pantothenate phosphorylation by one of two mechanisms: (i) by directly inhibiting *Pf*PanK, or (ii) by serving as alternate substrates that are competitively phosphorylated. It is noteworthy that the IC_{50} value of the best pantothenate phosphorylation inhibitor is 13-fold lower than the pantothenate concentration present in the assay (1.8 μM). Assuming competition for the same binding site, this is consistent with the enamide binding PanK with higher affinity and/or being turned over more slowly than the natural substrate. CJ-15,801 has been shown to be phosphorylated by the type II PanK of *S. aureus* [18]. As *P. falciparum* is also predicted to express a type II PanK [20] and has previously been shown to phosphorylate other pantothenate analogues [21,22], it seems likely that the observed inhibition of pantothenate phosphorylation is a consequence of the analogues serving as PanK substrates. Should this be true, the antiplasmodial effect could in turn be a consequence of phosphopantothenate depletion and/or the phosphorylated enamides acting as inhibitors of PPCS (the subsequent enzyme in the CoA biosynthesis pathway), as has been shown to be the case in *S. aureus* [18]. An alternate possibility is that the CoA biosynthesis pathway metabolizes the phosphorylated enamides further to produce CoA anti-metabolites that in turn kill the parasite. Bacterial CoA biosynthesis enzymes have previously been shown to metabolize amide analogues of pantothenate (pantothenamides) to yield CoA anti-metabolites [23]. The establishment of a reliable overexpression system for *Pf*PanK will greatly facilitate further investigations into the mechanism of action of enamide and other analogues of pantothenate. Based on the observed relationship between the effect of the ester analogues of CJ-15,801 on pantothenate phosphorylation and their antiplasmodial effect, it is tempting to speculate that the interaction with PanK is a key determinant of antiplasmodial activity. The observation that CJ-15,801 does not conform with the rest of the series, however, implicates an additional/alternate determinant (such as uptake and/or affinity of the phosphorylation-activated compound for PPCS). For example, as CJ-15,801 most closely resembles pantothenate than the ester analogues, its activated derivative(s) may bind the ultimate target with higher affinity, compensating for a lower rate of activation by PanK. Understanding why CJ-15,801 behaves as an outlier has implications for elucidating the mechanism of action of these and possibly other pantothenate analogues.

2.4 Susceptibility to esterase-mediated hydrolysis

The SAR observed for the ester analogues of CJ-15,801 in the pantothenate phosphorylation assay suggests that the ester moieties are not readily hydrolysed under the conditions of this assay (Figure 4). However, to investigate whether *R,E*-**1d** is subject to esterase-mediated hydrolysis within *P. falciparum*-infected erythrocytes, we synthesized *R,E*-**1d** (as well as CJ-15,801, for reference) with ^{13}C labels introduced into the enamide moiety. The labeled compounds were synthesized using $^{13}C_2$-bromoacetic acid (**8**) and ^{13}C-formic acid (**12**) as precursors (Schemes 3 and 4). *P. falciparum*-infected erythrocytes were incubated with ^{13}C-*R,E*-**1d** and, after 3 h, metabolites were extracted from the parasite. The extracts were then analyzed by ^{1}H-^{13}C-heteronuclear single quantum coherence (HSQC) NMR. Peaks corresponding to the olefinic carbons of *R,E*-**1d**, but not CJ-15,801, were observed (Figure 5), consistent with intact *R,E*-**1d** gaining access to the parasite and the ester moiety primarily being retained.

Scheme 3. Incorporation of ^{13}C-labeled building blocks into synthetic intermediates.

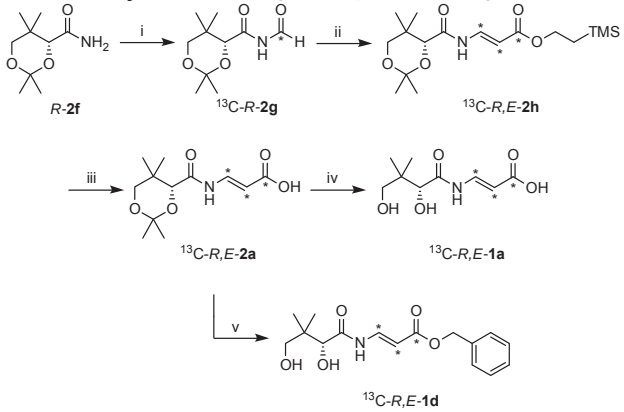


Reagents and conditions: (i) 2-(trimethylsilyl)ethanol, DCC, DMAP, DCM, 0 ˚C, 2 h, 68%; (ii) PPh_3, toluene, RT, 18 h, 65%; (iii) neat, 50 ˚C, 3 h, 70%; (iv) benzotriazole, THF, -10 ˚C, 1 h, 99%.

2.5 ^{1}H NMR analysis of compound reactivity

CJ-15,801 and analogues **1a-e** share an α,β-unsaturated carbonyl moiety that could potentially act as a Michael acceptor, and, consequently, react non-specifically with proteins [24]. The observed antagonistic effect of pantothenate (Figure 2), SAR observed among CJ-15,801 analogues that retain the *R*-

Scheme 4. Synthesis of $^{13}C_3$-CJ-15,801 and $^{13}C_3$-*R,E*-1d.



Reagents and conditions: (i) *n*-BuLi, THF, 0 ˚C, 0.5 h then **14**, 0 ˚C, 16 h, 78%; (ii) **10**, NaOH / DCM, RT, 2 h then $^{13}C_3$-*R*-**2g**, C_6H_6, 80 ˚C, 16 h, 97 %; (iii) TBAF, THF, 40 ˚C, 18 h, 80%; (iv) $BiCl_3$, aq. MeCN, RT, 16 h, 41%; (v) BnOH, DCC, DMAP, DCM, 0 ˚C, 4 h, 41%.

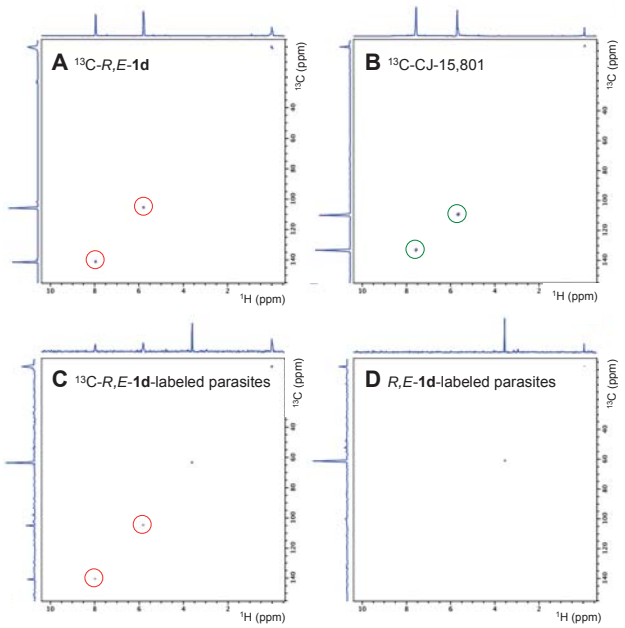


Figure 5. ^{1}H-^{13}C-HSQC analysis of metabolites extracted from parasites following a 3-h incubation of *P. falciparum*-infected erythrocytes with $^{13}C_3$-*R,E*-1d. ^{1}H-^{13}C-HSQC spectra of (**A**) $^{13}C_3$-*R,E*-**1d** (1 mM), (**B**) $^{13}C_3$-CJ-15,801 (1 mM), and extracts prepared from ~2 × 10^8 parasites after incubating *P. falciparum*-infected erythrocytes (> 95% parasitemia) for 3 h with $^{13}C_3$-*R,E*-**1d** (**C**) or unlabeled *R,E*-**1d** (**D**) are shown. The spectra shown in C and D are representative of those obtained in two independent experiments. All spectra were acquired in D_2O containing TSP (5 mM for A and B, or 200 µM for C and D) as an internal ^{1}H and ^{13}C chemical shift reference. The cross-peaks corresponding to the olefinic CH groups of $^{13}C_3$-*R,E*-**1d** and $^{13}C_3$-CJ-15,801 are circled in red and green, respectively.

pantoyl moiety (Table 1), and previous demonstration that after phosphorylation and cytidylylation CJ-15,801 acts as a tight-binding rather than irreversible inhibitor of *S. aureus* PPCS, provide strong evidence that the observed inhibitory activities (on parasite growth and pantothenate phosphorylation) are a result of a specific effect and not a result of the compounds reacting non-specifically. Nevertheless, we used ^{1}H NMR to investigate directly the reactivity of CJ-15,801 and *R,E*-**1d**. We were unable to detect any reaction between these compounds and glutathione or L-cysteine following a 2 h incubation at the pH (pH 7.4) used in the parasite growth and pantothenate phosphorylation assays (Supplementary information Figure S2). By contrast, under the same conditions, *N*-ethylmaleimide (a compound with a reactive Michael acceptor) reacted rapidly (before the first NMR spectrum was acquired 15 min after mixing with the thiols).

2.6 Selectivity

The selectivity of CJ-15,801 and its analogues was further explored by screening the compounds against two human cell lines: an immortalized cell line, namely, the human embryonic kidney (HEK-293) cell line, and a low-passage cell line (i.e. with a finite lifetime), namely, the human Caucasian fetal lung fibroblast WI-38 cell line. The effect of the enamides on proliferation of HEK-293 and WI-38 cells was assessed over 96 h, or 48 h, respectively. Favorably, the enamides with a pantothenate-dependent antiplasmodial effect (i.e. CJ-15,801 and ester analogues *R,E*-**1c-e**) showed little-to-no effect on the proliferation of HEK-293 or WI-38 cells at a concentration of 100 µM (≤ 5% and ≤ 36% inhibition, respectively) (Supporting information Table S1). The same was also true of the remaining pantoyl-bearing enamides tested. As the compounds did not inhibit proliferation of the cell lines by more than 50% at the highest concentration, we were unable to determine an absolute IC_{50} from which we could calculate absolute selectivity indices. Therefore, we instead divided the highest concentration of the enamides tested against human cells (at which proliferation was inhibited by < 50%) by the IC_{50} measured against the parasite, to obtain a lower limit for the selectivity index (Supporting information Table S1). The enamide with the greatest antiplasmodial effect (*R,E*-**1d**) was more than eight times more effective against the parasite.

The low cytotoxicity observed for the key antiplasmodial compounds (*R,E*-**1c-d**) is encouraging and consistent with the compounds not being generally toxic, and rather inhibiting parasite proliferation via a specific mechanism. In the future, it will be important to investigate the basis for the observed selectivity (e.g. by studying the effects on CoA biosynthesis in mammalian cells directly as done by Zhang *et al.* [25]) to ensure optimization only of molecules that also lack toxicity *in vivo*.

3. Conclusions

In conclusion, the *R*-pantoyl moiety and *trans*-substituted double bond geometry of CJ-15,801 was found to be critical for inhibition of pantothenate phosphorylation and an antiplasmodial effect that can be antagonized by pantothenate. The pantothenate concentration-independent effect of enamides without these key structural features is consistent with these compounds primarily inhibiting parasite proliferation through an unknown mechanism(s) unrelated to inhibition of pantothenate and/or CoA utilization. Replacement of the carboxylic acid with esters was tolerated and even led to improved inhibition of pantothenate phosphorylation and, for the most sterically-hindered ester analogue (*R,E*-**1d**), also led to increased antiplasmodial activity. These data suggest that future medicinal chemistry efforts should focus on additional carboxylic acid replacements as a means to improve activity.

Second generation analogues with improved activity should be tested not only for activity against the disease-causing asexual blood stage of *P. falciparum* (as done in this study) but also for activity against the liver stage, and the stage infective to mosquitoes (gametocytes), to investigate their potential as prophylactic and transmission blocking drugs, respectively. As the CoA-dependent fatty acid synthesis pathway is required for survival of liver stage parasites [26,27], inhibitors of CoA biosynthesis (pending the ability to permeate liver cells) are anticipated to be active against liver stage parasites. Fletcher *et al*. [28] have identified compounds structurally distinct from pantothenate analogues that inhibit growth of *P. falciparum* via an effect on CoA biosynthesis. Some of these molecules also inhibit early and late stage gametocytes, suggesting that other molecules that target CoA biosynthesis might also be effective against these forms of the parasite.

Appendix A. Supplementary data

Effect of enamides with a substituted double bond and/or pantoyl replacement on *P. falciparum* PanK-catalyzed pantothenate phosphorylation (Figure S1), ^{1}H NMR analysis of enamide reactivity (Figure S2), cytotoxicity of enamides against the human embryonic kidney (HEK-293) and human Caucasian fetal lung fibroblast (WI-38) cell lines (Table S1), experimental procedures, and ^{1}H and ^{13}C NMR spectra of the test compounds.

Author Contributions

All authors have given approval to the final version of the manuscript.

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The authors declare no competing financial interest.

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Abbreviations

CoA, coenzyme A; PanK, pantothenate kinase; *Pf*PanK, *P. falciparum* pantothenate kinase; SAR, structure-activity-relationship.

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- Synthesised new pantothenate analogues based of the natural product CJ-15,801.
- Identified analogues with on-target antiplasmodial activity and low toxicity.
- Determined structural requirements for on-target antiplasmodial activity.
- Linked antiplasmodial activity to inhibition of pantothenate phosphorylation.