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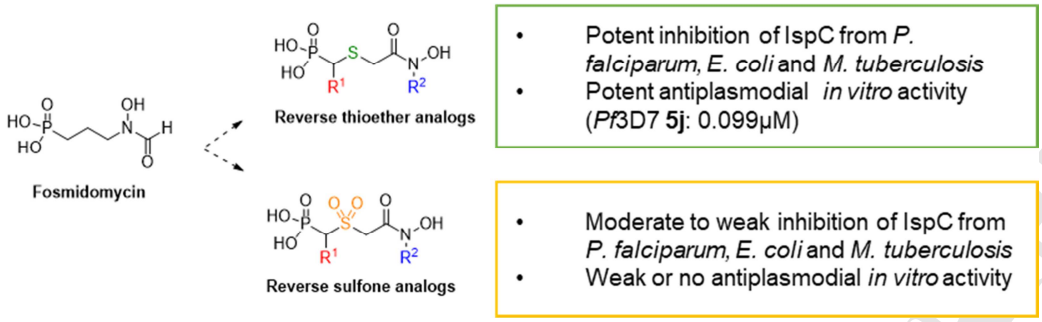
Graphical Abstract



Novel reverse thia-analogs of fosmidomycin: synthesis and antiplasmodial activity

Claudia Lienau[†], Tobias Gräwert,[§,¥] Leandro A. Alves Avelar,[†] Boris Illarionov,[§] Jana Held,[#] Tanja C. Knaab, [†] Beate Lungerich,[†] Lasse van Geelen,[¤] Dieter Meier,[¤] Stefanie Geissler,[‡] Holger Cynis [‡] Ulrich Riederer,[∂] Mirko Buchholz [‡] Rainer Kalscheuer,[¤] Adelbert Bacher,[§,&] Benjamin Mordmüller,[#] Markus Fischer,[§] Thomas Kurz[,†]*

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KEYWORDS. fosmidomycin thia isosters, sulfones, IspC, antiplasmodial activity, enantioselectivity

ABSTRACT. *β*-Thia analogs of fosmidomycin are potent inhibitors of the non-mevalonate isoprenoid biosynthesis enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IspC, Dxr) of *Plasmodium falciparum*. Several new thioethers displayed antiplasmodial *in vitro* activity in the low nanomolar range, without apparent cytotoxic effects in HeLa cells. The (*S*)-(+)-enantiomer of a typical representative selectively inhibited IspC and the growth of *P. falciparum* in continuous culture. The inhibitor was stable at pH 7.6 and room temperature, and no racemization was observed under these conditions during a period of up to two days. Oxidation of selected thioethers to sulfones reduced antiplasmodial activity and the inhibitory activity against *Escherichia coli*, *Mycobacterium tuberculosis* and *P. falciparum* IspC orthologs.

1. Introduction

Malaria continues to be a major cause of morbidity and mortality, particularly in Sub-Saharan Africa. Among human-pathogenic *Plasmodium* species, *Plasmodium falciparum* is the clinically most relevant and responsible for almost all fatal cases. Almost half of the world's population is at risk and an estimated 435,000 deaths occurred in 2017, most of them in early childhood. Infections and reinfections were estimated at 219 million cases for the year 2017.[1] Malaria control and therapy is an expensive and complex undertaking. Resistance of the pathogens and its vectors against many antimalarials and insecticides is now widespread and progressing further. Even the efficacy of artemisinins, nowadays the most used class of drugs, is jeopardized by emerging of new resistance mechanisms.[2,3] In the last years, many reports appeared about development of antimalarial vaccines, one undergoing implementation trials in selected areas, though with moderate success.[4,5] There is consensus in the expert community that new antimalarial drugs need to be introduced in the next decade if malaria is to be contained.[6]

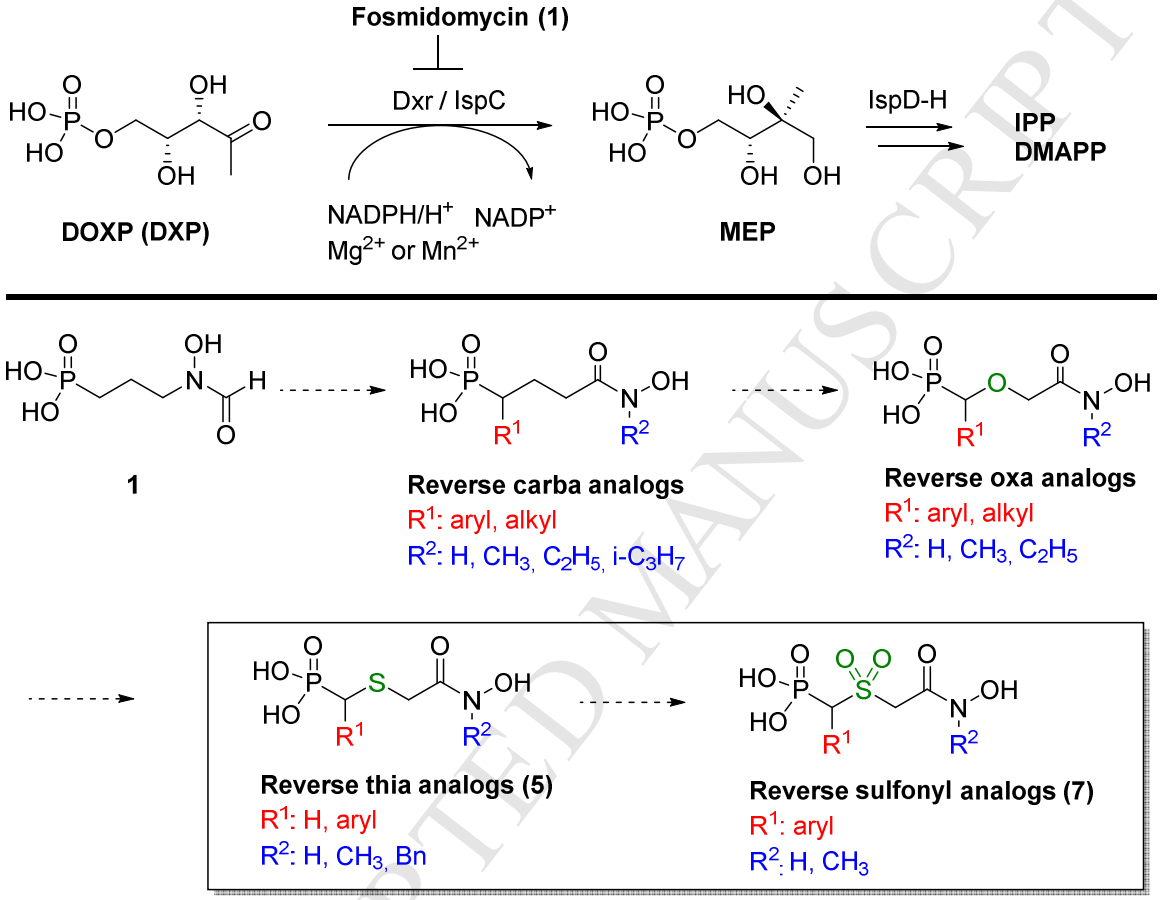
Plasmodia depend on the synthesis of isoprenoids via the non-mevalonate pathway that was elucidated in the late 1990s.[7–9] The non-mevalonate isoprenoid biosynthesis pathway is present in protozoa, eubacteria and plants but is absent in humans, therefore inhibitors of the parasite's isoprenoid biosynthesis enzymes should be exempt from target-related toxicity. Fosmidomycin (**1**) is a hydroxamic acid natural product that was discovered as an antibacterial agent in the late 1970s.[10] In 1999 it was shown that **1** inhibits 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IspC, Dxr) of *P. falciparum*, the enzyme catalyzing the first committed step of the non-mevalonate pathway.[8] The combination of fosmidomycin and piperaquine was very recently shown to be effective and well tolerated in a phase II clinical trial in the treatment of uncomplicated *P. falciparum* malaria.[11] However, despite these promising preliminary results, fosmidomycin has hitherto not been implemented as a marketed antimalarial drug. This is due to its pharmacokinetic and pharmacodynamic profile that only partially matches the currently preferred target product profile.[12] Several research groups have

therefore engaged in the development of fosmidomycin derivatives with improved properties.[13-30]

Fosmidomycin's mode of action is based on chelation of a divalent metal cation at the IspC active site, which is crucially involved in the transformation of 1-deoxy-D-xylulose 5-phosphate, (DOXP, DXP) into 2-*C*-methyl-D-erythritol 4-phosphate (MEP). MEP is a key intermediate in the biosynthesis of the essential isoprenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Figure 1).[31,32] Attempts to replace the metal-chelating hydroxamate group and the phosphonate group of **1** by (bio)isosteric functionalities have led to limited success against IspC orthologs of *Mycobacterium tuberculosis* and *Escherichia coli*.[33] However, retro-inversion and methylation of fosmidomycin's hydroxamate motif afforded derivatives with improved inhibitory properties (Figure 1).[15] Other important modifications were the introduction of α-(hetero)aryl, α-fluoro and β-arylalkyl side chains and the isosteric replacement of methylene groups. [33,34]. Furthermore, several types of phosphonate prodrugs with in part improved antiplasmodial activity have been reported.[23,28,30]

Notably, an α-phenyl β-thia isoster showed strong enantioselectivity; specifically, the inhibitory activity of the (*S*)-enantiomer exceeded that of the (*R*)-enantiomer by at least three orders of magnitude.[18] To get additional insights into the SAR of this series of reverse fosmidomycin analogs we synthesized and tested four novel 3,5-diphenyl-substituted analogs (**5e,f,h,i**), two new para-substituted thioethers (**5g,j**) and for comparison their cognate sulfones (**7**).

Figure 1. IspC inhibition and reverse fosmidomycin analogs.

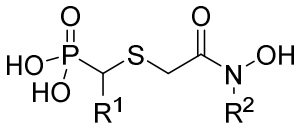


2. Results

2.1. Synthesis of novel α-phenyl β-thia isosters

To further explore the influence of substituents at the phenyl ring of **5a**, the synthesis of six novel structural analogs **(5e-j)** using the previously published strategy was performed (Scheme 1, Table 1).[18] To assess their inhibitory activity the novel thioethers **5e**-**5j** were first tested for inhibition of IspC from *Escherichia coli*, *Mycobacterium tuberculosis* and *Plasmodium falciparum* using a photometric assay. IspC inhibition data shown in Table 1 include the novel compounds **5e**-**5j** and for comparison the previously published compounds (**5a-d**).

Table 1. IC_{50} values of thioethers versus IspC orthologs from *P. falciparum, E. coli and M. tuberculosis*.



	R^1	R^2	IspC *P. falciparum* IC_{50} [µM][a]	IspC *E. coli* IC_{50} [µM][a]	IspC *M. tuberculosis* IC_{50} [µM][a]
5a[b]	Ph	CH_3	0.024±0.003	0.0082±0.0008	0.28±0.03
5b[b]	4-CH_3-Ph	CH_3	0.018±0.004	0.033±0.009	0.11±0.01
5c[b]	naphth-1-yl	CH_3	0.0098±0.0009	0.14±0.01	0.55±0.01
5d[b]	3,4-Cl-Ph	CH_3	0.0045±0.0004	0.0059±0.0006	0.0092±0.0006
5e	3,5-F-Ph	CH_3	0.013±0.004	0.018±0.003	0.11±0.01
5f	3,5-OCH_3-Ph	CH_3	0.020±0.004	0.16±0.03	4.4±0.2
5g	4-CH_3-Ph	H	0.032±0.006	0.29±0.02	8.2±0.5
5h	3,5-OCH_3-Ph	H	0.024±0.004	0.68±0.13	44±4
5i	3,5-F-Ph	H	0.011±0.002	0.013±0.001	0.32±0.03
5j	4-SCH_3-Ph	CH_3	0.029±0.008	0.022±0.005	0.10±0.02
Fosmidomycin			0.16±0.02	0.22±0.01	0.23±0.02

[a]Photometric assay.[18,35] Values are mean ± standard deviation. In general, two to three independent determinations were performed. [b]IC_{50} values for this compound have been published before.[18]

In line with earlier results, the novel thioethers **5e**-**5j** inhibited IspC from *P. falciparum* with IC_{50} values in the low nanomolar range. With IC_{50} values ranging from 0.0045 – 0.032 µM for the racemates, the structure activity relationship in the group of ten structural analogs is relatively flat (Figure 2).

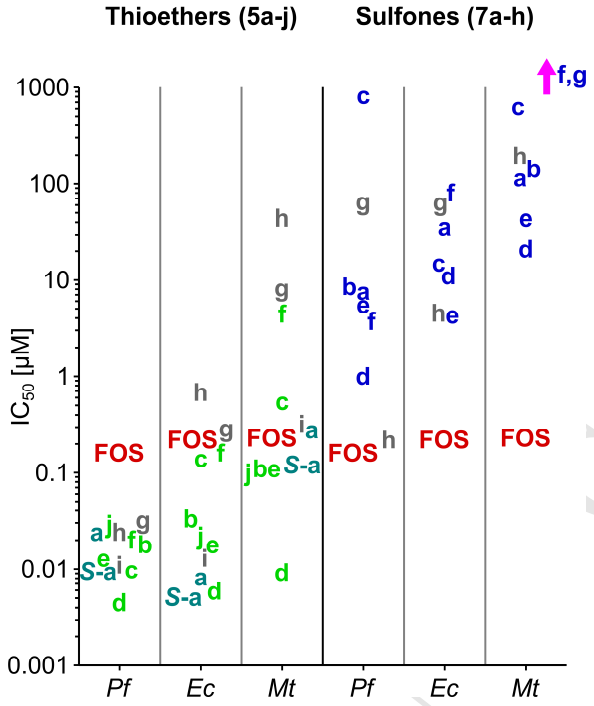


Figure 2. Activity (IC_{50}) of thioether compounds **5a-j** in comparison to their cognate sulfones **7a-h**. Both types of compounds inhibit IspC enzyme from *P. falciparum*, *E. coli* and *M. tuberculosis*. Red: Fosmidomycin. Light/dark green: Thioether compounds with R^2=CH_3. Dark green: Compound **5a** in its racemic form (a) and as the active enantiomer ((*S*)-5a). Grey: Thioether and Sulfone compounds with R^2=H. Blue: Sulfone compounds with R^2=CH_3. Pink arrow: IC_{50} greater than 1000 µM.

On the other hand, the IC_{50} values observed with IspC of *E. coli* (0.0059 – 0.68 µM) resp. *M. tuberculosis* (0.0092 µM – 44 µM) span a considerably wider range, in comparison with the *Plasmodium* enzyme (0.0045 – 0.032 µM). Both bacterial enzymes are less sensitive to these inhibitors than the *Plasmodium* enzyme. Notably, however, the strongest inhibitor, the 3,4-dichloro-*N*-methyl analogue **5d** inhibited all three enzymes with IC_{50} values in the single-digit nanomolar range (values of 4.5 to 9.2 nM).

*2.2. Antiplasmodial in vitro activity of novel α-phenyl β-thia isosters (**5e-j**).*

We next compared the antiplasmodial in vitro activity of earlier compounds and newly synthesized compounds. Novel analogs **5e**, **5f**, **5h** and **5j** displayed nanomolar *in vitro* antiplasmodial activity along with absence of cytotoxicity toward HeLa cells, even at

millimolar concentrations (Table 2). However, the new compounds **(5e-j)** are somewhat less active than the previously reported derivatives (**5a-d**). In line with the enzyme assays, the *N*-methyl substituted derivatives demonstrated stronger antiplasmodial activity toward strains 3D7 and Dd2 than the unsubstituted hydroxamic acids. The most active novel thioethers (**5e**, **j**) showed IC_{50} values of 0.15 µM (**5e**) and 0.099 µM (**5j**) against the *Plasmodium* 3D7 strain. However, compared to their inhibitory activity at the enzyme level toward *Pf*IspC the antiplasmodial in vitro activity of racemic thioethers **5a-j** is weaker (Table 1 vs Table 2). Since no stability problem has been observed (Figure 3), this is most likely due to the fact that the *Pf*IspC inhibitors have to penetrate several membranes in order to bind to the active site of *Pf*IspC in the apicoplast. Table 2 also shows that the chloroquine resistance has not much influence on the antiplasmodial activity of compounds **5**.

Table 2. IC_{50} values of thioethers **5a-j** versus *Plasmodium* strain 3D7, Dd2 and versus HeLa cells.

	R^1	R^2	*Pf*3D7 IC_{50} [µM][a]	*Pf*Dd2 IC_{50} [µM][a]	HeLa IC_{50} [µM][a]	SI[b]
5a[c]	Ph	CH_3	0.19±0.08	0.26±0.05	> 1000	>37000
5b[d]	4-CH_3-Ph	CH_3	0.11±0.04	0.095±0.041	n.d.	n.c.
5c[d]	naphth-1-yl	CH_3	0.38±0.07	0.51±0.18	> 1000	>2600
5d[d]	3,4-Cl-Ph	CH_3	0.091±0.076	0.085±0.030	> 1000	>11000
5e	3,5-F-Ph	CH_3	0.15±0.06	0.13±0.07	> 1000	>6700
5f	3,5-OCH_3-Ph	CH_3	0.16±0.01	0.18±0.04	> 1000	>6400
5g	4-CH_3-Ph	H	6.5±2.8	8.0±2.3	> 1000	>150
5h	3,5-OCH_3-Ph	H	0.33±0.17	0.27±0.13	> 1000	>3000
5i	3,5-F-Ph	H	1.2±0.5	0.87±0.18	> 1000	>810
5j	4-SCH_3-Ph	CH_3	0.099±0.060	0.18±0.003	> 1000	>10000
Fosmidomycin			0.88±0.18	0.81±0.16	n.d.	-

[a]*In vitro* assay. Values are the mean of two independent double determinations. [b]Selectivity Index (SI) shown as HeLa IC50/*Plasmodium* IC50 for strain 3D7. Values larger than 1 indicate greater parasite selectivity. [c] Previously published[18] values for *Pf*3D7 (0.030 µM) and *Pf*Dd2 (0.075 µM) are herewith revised. [d]IC_{50} values for this compound have been published earlier.[18] n.d., not determined. n.c., not calculated.

2.3. Compound stability and absence of racemization.

Based on earlier results, it's promising activity and synthetic accessibility compound **5a** was chosen to be further studied. First, the stability of **5a** in phosphate buffer pH 7.6 was monitored by HPLC over a period of 72 h and almost no decomposition was observed (>95% drug content). No net loss of inhibitory activity of racemic **5a** occurred over a period of two days (Fig. 3). In order to study potential racemization, (*S*)- resp. (*R*)-enantiomer of **5a** were incubated in 100 mM Tris hydrochloride, pH 7.6.

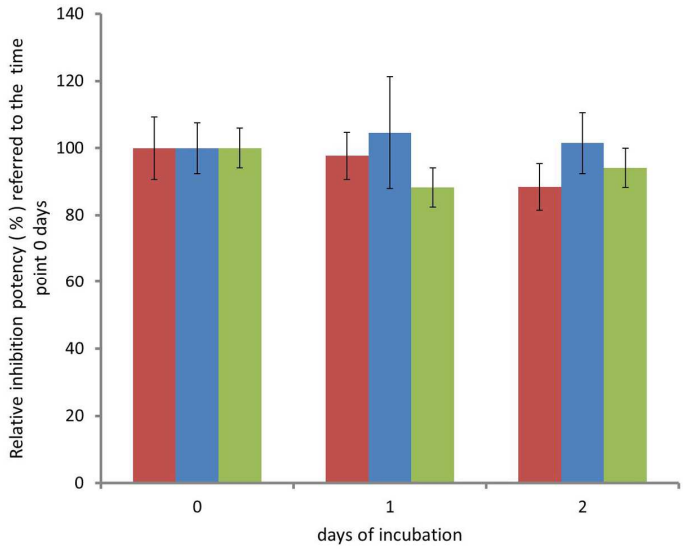


Figure 3. Stability of **5a** in course incubated in phosphate buffer pH 7.6 at room temperature (in % of the IC_{50} value ± SD for racemate or the respective enantiomer at the beginning of incubation). Red, racemate; blue, (*R*)-**5a**, green, (*S*)-**5a**.

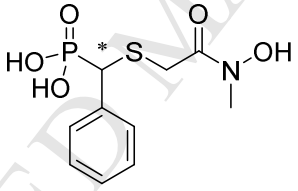
Since any racemization of the inactive (*R*)-enantiomer should generate the active (*S*)-enantiomer, the inhibitory potency of the (*R*)-**5a** sample should increase with time. Any significant formation of the inactive (*R*)-enantiomer from the active (*S*)-enantiomer

should have resulted in a decrease of the apparent IC_{50} value of the (*S*)-**5a** sample (i.e. a decrease of enzyme inhibition by generation of the inactive enantiomer). However, apparent IC_{50} values for *P. falciparum* IspC determined at intervals with (*S*)- respectively (*R*)-enantiomer of **5a** in solution did not change significantly, neither over a period of two days at room temperature (Fig. 3) nor over a period of seven days at +5 °C (data not shown).

2.4. Enantioselectivity in the P. falciparum replication assay.

Earlier, we had shown that the IspC orthologs from *P. falciparum, E. coli,* and *M. tuberculosis* showed high enantiospecificity for the (*S*)-(+)-enantiomer of **5a**. [18] Now we studied their enantioselectivity in the *P. falciparum* replication assay. The enantiomers of **5a** showed a high degree of enantioselectivity as determined by replication of *P. falciparum* in human erythrocytes (Figure 4, Table 3).

Table 3. Antiplasmodial *in vitro* activity of **5a** racemate and enantiomers



5a (racemate)

	*Pf*3D7 IC_{50} [µM][a]	*Pf*Dd2 IC_{50} [µM][a]
5a racemate[b]	0.19 ± 0.08	0.26 ± 0.05
(*S*)-(+)-**5a**	0.087 ± 0.033	0.14 ± 0.05
(*R*)-(-)-**5a**	>130	>130
Fosmidomycin	0.88±0.18	0.81±0.16

[a]Values are the mean of two independent double determinations. [b] Previously published [19] values for *Pf*3D7 (0.030 µM) and *Pf*Dd2 (0.075 µM) are herewith revised.

Figure 4 compares the enantioselectivity observed in the enzyme assay and the parasite replication assay. In the enzyme assay as well as the parasite assay, the (*S*)-enantiomer has approximately double potency by comparison with the racemate. In both assay systems, the activity of the (*S*)-enantiomer is lower than that of the racemate by at least three orders of magnitude. Notably, the dose-response curves in the parasite assay show a much steeper slope as compared to the enzyme assay.

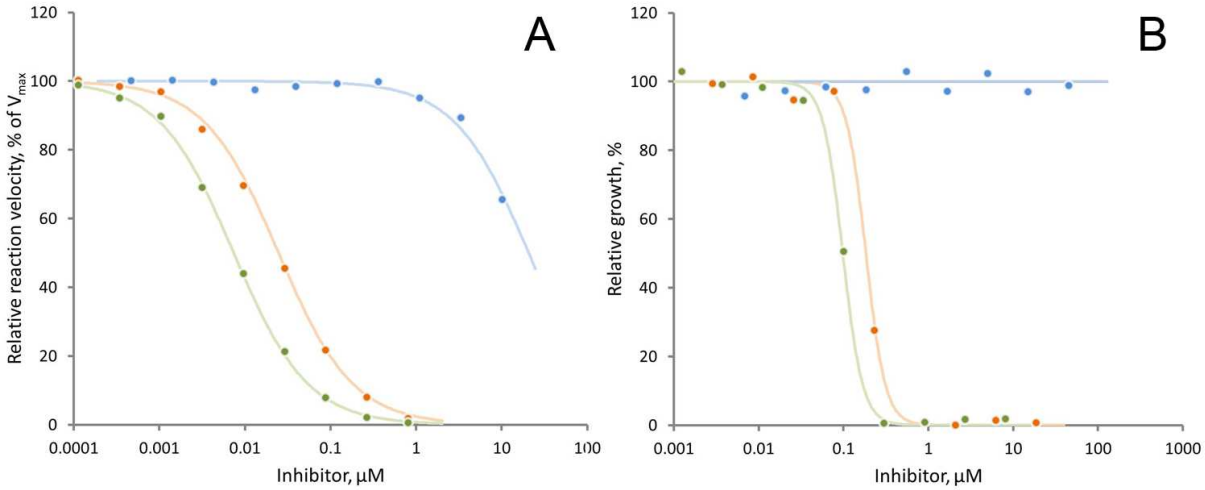


Figure 4. Dose response curves. **A**, photometric assay of *P. falciparum* IspC; **B**, *P. falciparum* asexual blood stages assay. green, (*S*)-(+)-**5a**; blue, (*R*)-(-)-**5a**; orange, racemic **5a**.

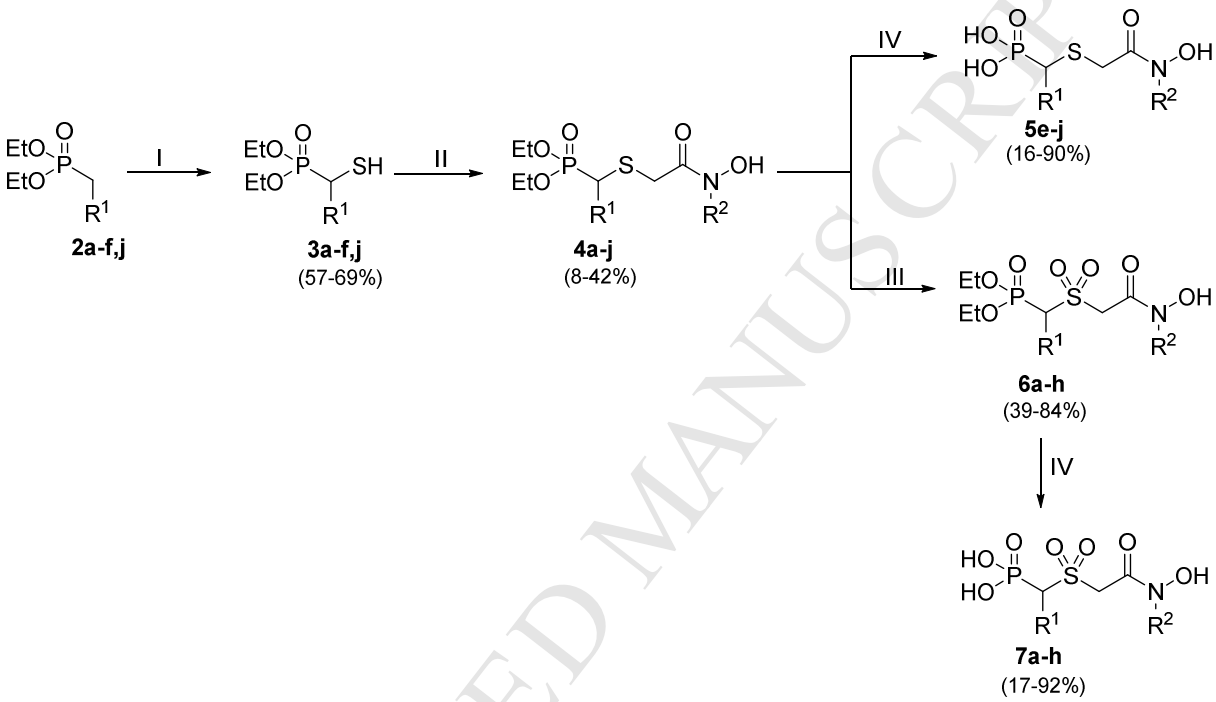
2.5. Conversion of thioethers to sulfones.

Based on the X-ray structure of *Pf*IspC in complex with (*S*)-(+)-**5a** (PDB ID 4KP7) [18], we performed a modeling study. [36] in order to analyze whether the active site of the *Plasmodium* enzyme could accommodate derivatives of **5a** with a higher oxidation level of the sulfur (Figure 1A, SI). The sulfone analog **7a** of thioether **5a** could be accommodated inside the active site without steric clashes.

Several sulfone derivatives (**7a-h**) were obtained in good yields (Scheme 1). Specifically, diethyl phosphonates **4a-h** were accessible via *S*-alkylation of the respective α-mercaptophosphonates **3a-f**, **j** as published before. [18] Novel diethyl phosphonates **4e-j** were deprotected by treatment with TMSBr and yielded thioethers

5e, f, j as solids and **5g-i** as oils.[37] Oxidation of diethyl phosphonates **4a-h** with 3-chlorobenzoperoxoic acid provided the partially protected sulfones **6a-h**. [38] The diethyl phosphonate moieties of intermediates **6a-h** were deprotected with TMSBr and afforded sulfones **7a-g** as oils and **7h** as white solid.

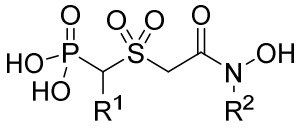
Scheme 1. Synthesis of thioethers **5e-j** and sulfones **7a-h**.



Reagents and conditions. I: *n*-BuLi, S, THF, -78 °C, 1 h; 2. -20 °C, THF, 1 h; 3. RT, THF, 1 h; II: **4a-f, j**: 2-chloro-*N*-hydroxy-*N*-methylacetamide, DMF, Na_2CO_3, 0 °C → RT; **4g-i**: 2-chloro-*N*-hydroxyacetamide, DMF, Na_2CO_3, 0 °C → RT; III: 3-chlorobenzoperoxoic acid, CH_2Cl_2, 0 °C, 1h; CH_2Cl_2, RT, 12 h; IV: 1. TMSBr, CH_2Cl_2, 0 °C, 1 h; 2. CH_2Cl_2, RT, 47 h; 3. THF/H_2O, RT, 45 min. The synthesis of **2a-d** and **5a-d** has been described previously.[18]

Inhibitory activities of the sulfone derivatives (**7a-h**) for IspC from *P. falciparum*, *E. coli* and *M. tuberculosis* were determined using the photometric assay (Figure 4, Table 3). [20] Data were collected at a substrate (DOXP) concentration of 500 µM.

Table 3. IC_{50} values of sulfones **7a-h** versus IspC orthologs from *P. falciparum*, *E. coli* and *M. tuberculosis*.



	R^1	R^2	IspC *P. falciparum* IC_{50} [µM][a]	IspC *E. coli* IC_{50} [µM][a]	IspC *M. tuberculosis* IC_{50} [µM][a]
7a	Ph	CH_3	7.6±0.7	36±2	115±6
7b	4-CH_3-Ph	CH_3	8.5±0.9	36±3	143±23
7c	naphth-1-yl	CH_3	823±164	15±0	621±83
7d	3,4-Cl-Ph	CH_3	1.0±0.2	11±1	21±1
7e	3,5-F-Ph	CH_3	5.5±0.3	4.3±0.3	44±4
7f	3,5-OCH_3-Ph	CH_3	3.7±0.6	80±5	>1000
7g	4-CH_3-Ph	H	65±9	64±7	>1000
7h	3,5-OCH_3-Ph	H	0.22±0.03	4.5±0.3	194±12
Fosmidomycin			0.16±0.02	0.22±0.01	0.23±0.02

[a]Enzyme assay. Values were calculated from at least eight data points. In general, two to three independent determinations have been performed.

Without exception, the sulfone derivatives **7a-h** are weaker IspC inhibitors as compared to their cognate thioether analogs (Figures 2, 5). Typically, the oxidation of the thioether motif to the sulfone level decreased the inhibitory activity by 2 to 3 orders of magnitude.

A notable exception occurred with the 3,5-dimethoxy compounds. The loss of inhibitory activity of **7h** in comparison with **5h** is less than 1 order of magnitude for all three enzyme orthologs.

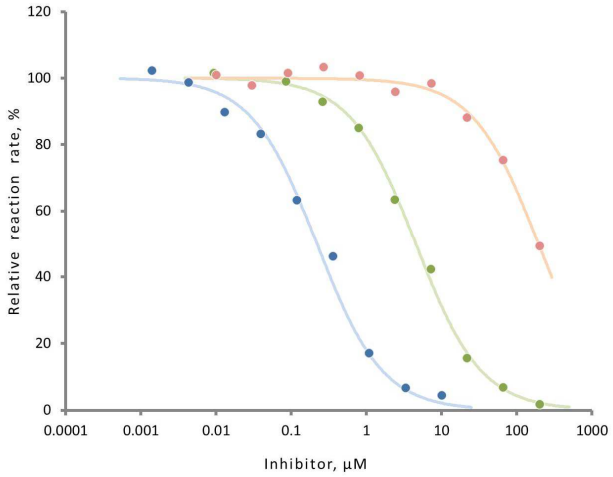


Figure 5. Inhibition of IspC orthologs (*P. falciparum*, blue; *E. coli*, green; *M. tuberculosis*, orange) with sulfone **7h**.

In contrast to their highly active cognate thioether analogs **5a-j**, the sulfones **7a-d, f, g** showed little or no antiplasmodial in vitro activity toward *Pf*3D7 up to a concentration of 100 µM. Only sulfone **7h** displayed at least some antiplasmodial activity with an IC_{50} 2.7 µM.

Table 4. IC_{50} values of sulfones **7a-h** versus *Plasmodium* strain 3D7 and Dd2.

	R^1	R^2	*Pf*3D7 IC_{50} [µM][a]	HeLa IC_{50} [µM][a]
7a	Ph	CH_3	>100	n.d.
7b	4-CH_3-Ph	CH_3	>100	> 50
7c	naphth-1-yl	CH_3	>100	> 1000
7d	3,4-Cl-Ph	CH_3	>100	> 1000
7e	3,5-F-Ph	CH_3	19± 3	> 1000
7f	3,5-OCH_3-Ph	CH_3	>100	> 1000
7g	4-CH_3-Ph	H	>100	n.d.
7h	3,5-OCH_3-Ph	H	2.7±0.4	> 500
Fosmidomycin			0.88±0.18	n.d.

[a]*In vitro* assay. Values are the mean of two independent double determinations. n.d., not determined.

2.6. Caco2 cell permeability assay.

To investigate the assumption of an insufficient transport through membranes in the *P. falciparum* in vitro *growth* inhibition assays of **5a** and **5j** a Caco2-cell permeability assay was performed. This assay is used in preclinical drug development for the assessment of a compound transport through epithelial cell barriers, especially in the GI-tract. After administration of the compounds to the polarized cell monolayer, the concentration of the compound is measured time dependently on both sides, the donor and acceptor medium, as well as in the cells themselves by using LC-MS/MS.[39] As a result for both compounds very low P_{app}-values were calculated with even smaller values than for atenolol, a compound used as a negative control in this assay.

Table 6. Caco2 cell assay of **5a** and **5j**

	P_{app} [cm/s][a]
Atenolol[b]	$0.45 \cdot 10^{-6} \pm 0.02 \cdot 10^{-6}$
5a	$0.14 \cdot 10^{-6} \pm 0.01 \cdot 10^{-6}$
5j	$0.15 \cdot 10^{-6} \pm 0.01 \cdot 10^{-6}$

a Apparent permeability coefficient. [b]Atenolol is used as a reference for substances with a low permeability

2.7. In vitro antibacterial activity.

Based on their inhibition of *Ec*IspC and *Mt*IspC (Table 1 and 2), thioethers and sulfones were evaluated for antibacterial activity (Table 2, SI). With the exception of **5d**, no antibacterial activity was observed against *M. tuberculosis* and against a panel of Gram-negative bacterial strains using concentrations up to 100 µM. Compound **5d** showed very weak activity against a multidrug-resistant strain of *Acinetobacter baumannii* (MIC

= 100 µM; Table 2, SI). Thus, the high *in vitro* activity of thioethers **5** against purified *Ec*IspC and *Mt*IspC did not translate into antibacterial whole cell activity, which suggests that poor cell penetration rate could be the limiting factor.

3. Discussion

The current antimalarial drug development pipeline urgently requires novel antiplasmodial compounds.[40] The non-mevalonate isoprenoid biosynthesis is of particular interest, because its enzymes are absent in humans. The interaction of the first committed enzyme of the pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerases (IspC, Dxr), with the antibiotic fosmidomycin and its derivatives has been addressed in numerous publications.[33] The introduction of aryl substituents into the fosmidomycin skeleton has afforded compounds that showed significantly enhanced inhibition of *Pf*IspC, while also generating a chiral center.[34] X-ray structure analysis had suggested preferential binding of the (*S*)-enantiomers of α-substituted reverse fosmidomycin derivatives. Chromatographic enantiomer resolution of the α-phenyl β-thia analog **5a** has conclusively shown that the enantiomer selectivity of IspC from *P. falciparum* exceeds three orders of magnitude.

In an earlier study, we have shown explicitly that the IspC orthologs from *M. tuberculosis* and *E. coli* are also selectively inhibited by the (*S*)-(+)-enantiomer of **5a**, albeit at lower levels of enantioselectivity.[18] More importantly, we have now shown that enantioselectivity also extends to the antiplasmodial activity against growth of *P. falciparum* strains 3D7 and Dd2 (Table 3). The activity of the (*S*)-enantiomer exceeds that of the (*R*)-enantiomer by at least 3 orders of magnitude, thus supporting the view that IspC is the relevant target of fosmidomycin. The use of pure enantiomers would then effectively double the efficacy of the compounds, while simultaneously avoiding the risk of any potential toxicity effects of the inactive enantiomer.

Since no steric clashes were observed in the *in silico* studies with the sulfone derivatives, the different electronic properties of the sulfone moiety compared to the thioether group are one possible explanation for the decrease in the enzymatic and cellular activities. Whereas the most potent type **5** compounds outperformed

fosmidomycin by almost two orders of magnitude at the level of the molecular target, only an advantage of about one order of magnitude remains at the level of the *P. falciparum* growth inhibition assay. Inside the Plasmodium-infected erythrocyte, the molecular target of fosmidomycin is shielded by not less than 7 membrane barriers (the outer membrane of the erythrocyte, the membrane of the vacuole that contains the parasite, the outer Plasmodium membrane and 4 membranes of the apicoplast). In light of that extremely complex topology, it is not surprising that the IC_{50} value for each of the study compounds is larger than the IC_{50} for the isolated enzyme.

4. Conclusions

Reverse thioether analogs of fosmidomycin are highly active inhibitors of 1-deoxy-D-xylulose 5-phosphate reductoisomerases (IspC, Dxr) of *Plasmodium falciparum*, *Escherichia coli*, and *Mycobacterium tuberculosis.* Several IspC inhibitors exert antiplasmodial *in vitro* activity in the low nanomolar range without demonstrating cytotoxic effects in HeLa cells even at millimolar concentrations. The (*S*)-(+)-enantiomer of thioether **5a** selectively inhibited IspC and the growth of *P. falciparum* in continuous culture. None of the thioethers exhibited significant antibacterial *in vitro* activity against *M. tuberculosis* and a spectrum of Gram-negative bacteria up to a concentration of 100 µM. Oxidation of selected thioethers to sulfones reduced the antiplasmodial activity and the inhibitory activity toward *E. coli*, *M. tuberculosis* and *P. falciparum* IspC orthologs. Currently our work focuses on phosphonamidate prodrugs as a promising new class of phosphonate prodrugs and on reverse bissubstrate inhibitors, which target the substrate and NADPH binding site of IspC.

5. Experimental section

5.1. Chemistry

All solvents and chemicals were used as purchased without further purification. The progress of all reactions was monitored on Merck precoated silica gel plates (with fluorescence indicator UV_{254}). Column chromatography was performed with Fluka silica gel 60 (230-400 mesh ASTM) with the solvent mixtures specified in the corresponding experiment. Spots were visualized by irradiation with ultraviolet light (254 nm). Melting

points (m.p.) were taken in open capillaries on a Stuart melting point apparatus SMP11 and are uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance 500 (500.13 MHz for ^{1}H; 125.76 MHz for ^{13}C) using DMSO-d_6 as solvent. Chemical shifts are given in parts per million (ppm), (δ relative to residual solvent peak for ^{1}H and ^{13}C). Elemental analysis was performed on a Perkin Elmer PE 2400 CHN elemental analyzer and a vario MICRO cube elemental analyzer, Elementar Analysensysteme GmbH, Hanau Germany. Optical rotation was determined by a Krüss P8000 polarimeter; specific rotations $[\alpha]_D^{20}$ values are given in deg dm^{-1} cm^3 g^{-1}. High-resolution mass spectrometry (HRMS) analysis was performed using a UHR-TOF maXis 4G instrument (Bruker Daltonics, Bremen, Germany). If necessary, purity of compounds was determined by high performance liquid chromatography (HPLC). The purity of all novel final compounds (**5e-j**, **7a-h**) was 95% or higher. Instrument: Elite LaChrom system [Hitachi L-2130 (pump) and L-2400 (UV-detector)]; column: Phenomenex Luna C-18(2) 5 µm particle size (250 mm × 4.6 mm), supported by Phenomenex Security Guard Cartridge Kit C18 (4.0 mm × 3.0 mm).

5.1.1. Experimental data

*5.1.1.1. General procedure for the synthesis of phosphonic acids **5e-j** and **7a-h:***

The synthesis of **5a-d** has been reported previously by Kunfermann et al.[18] The appropriate phosphonic acid diethyl ester **4a-j**, **6a-h** (1eq., 1 mmol) was dissolved in dry dichloromethane (10 mL) under an atmosphere of nitrogen. The solution was cooled to 0 °C and trimethylsilyl bromide (5 eq., 0.66 mL, 5 mmol) was added dropwise via a syringe. After stirring for 1 h, the reaction mixture was allowed to warm up to RT and was stirred for further 47 h. The solvent was removed under reduced pressure and tetrahydrofurane (10 mL) was added to the residue. Next, the solution was treated with water (3 eq., 0.05 mL, 3 mmol) and the reaction mixture was stirred for 45 min at RT. Afterwards the solvent was evaporated and the residue was dried *in vacuo* overnight. Addition of ethyl acetate afforded phosphonic acids **5e, f, j** and **7h** as solid compounds. Target compounds **5g-i** and **7a-g** were obtained as oils after purification of the crude products by column chromatography with a small amount of silica gel using ethyl acetate/*n*-hexane (0:100 → 100:0).

5.1.1.2. ((3,5-Difluorophenyl)((2-(hydroxy(methyl)amino)-2-oxoethyl)thio)methyl) phosphonic acid **(5e):** white solid; yield: 60%. M.p. 135 °C; ^{1}H NMR (500.13 MHz, DMSO-d_6): δ=10.01 (br. s, OH), 7.17-7.12 (m, 1H, $H_{arom.}$), 7.09 (d, ^{3}J(H,H)=8.3 Hz, 2H, $H_{arom.}$), 4.33 (d, ^{2}J(H,P)=19.8 Hz, 0.87H, PCH), 4.25 (d, ^{2}J(H,P)=19.8 Hz, 0.13H, PCH), 3.48 (d, ^{2}J(H,H)=14.6 Hz, 1H, SCH_2), 3.28 (d, ^{2}J(H,H)=14.7 Hz, 1H, SCH_2), 3.09 (s, 0.31H, NCH_3), 3.05 (s, 2.53H, NCH_3), 3.01 ppm (s, 0.16H, NCH_3); ^{13}C NMR (125.76 MHz, DMSO-d_6): δ=168.5 (C=O), 161.8 (dd, ^{1}J(C,F)=246.8 Hz, ^{3}J(C,F)=13.0 Hz, $C_{arom.}$), 142.5-142.3 (m, $C_{arom.}$), 112.3 (dd, ^{2}J(C,F)=25.7 Hz, ^{4}J(C,F)=^{3}J(C,P)=5.7 Hz, $C_{arom.}$), 102.7-102.2 (m, $C_{arom.}$), 45.4 (d, ^{1}J(C,P)=137.5 Hz, PC), 35.7 (NCH_3), 32.0 ppm (d, ^{3}J(C,P)=7.8 Hz, SCH_2); HPLC analysis: retention time=1.887 min, peak area: 99.15%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.3. ((3,5-Dimethoxyphenyl)((2-(hydroxy(methyl)amino)-2-oxoethyl)thio)methyl) phosphonic acid **(5f):** yellow solid; yield: 18%. M.p. 108 °C; ^{1}H NMR (500.13 MHz, DMSO-d_6): δ=10.02 (br. s, OH), 6.61-6.55 (m, 2H, $H_{arom.}$), 6.42-6.36 (m, 1H, $H_{arom.}$), 4.16 (d, ^{2}J(H,P)=19.1 Hz, 1H, PCH), 3.72 (s, 6H, OCH_3), 3.50 (d, ^{2}J(H,H)=14.6 Hz, 1H, SCH_2), 3.27 (d, ^{2}J(H,H)=14.6 Hz, 1H, SCH_2), 3.07 ppm (s, 3H, NCH_3); ^{13}C NMR (125.76 MHz, DMSO-d_6): δ=168.6 (C=O), 159.9-159.8 (m, $C_{arom.}$), 139.6 (d, ^{5}J(C,P)=1.8 Hz, $C_{arom.}$), 107.5 (d, ^{3}J(C,P)=6.0 Hz, $C_{arom.}$), 98.5 ($C_{arom.}$), 55.1-54.9 (m, OCH_3), 45.9 (d, ^{1}J(C,P)=139.0 Hz, PC), 35.72 (NCH_3), 32.1-31.9 ppm (m, SCH_2); HPLC analysis: retention time=1.887 min, peak area: 99.57%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.4. (((2-(Hydroxyamino)-2-oxoethyl)thio)(p-tolyl)methyl)phosphonic acid **(5g):** orange oil; yield: 90%. ^{1}H NMR (600.22 MHz, DMSO-d_6): δ=10.54 (br. s, OH), 10.07 (br. s, OH), 10.03 (br. s, NH), 9.88 (br. s, NH), 7.27 (dd, ^{3}J(H,H)=8.2 Hz, ^{4}J(H,H)=1.5 Hz, 1H, $H_{arom.}$), 7.25 (d, ^{3}J(H,H)=7.9 Hz, 1H, $H_{arom.}$), 7.11 (d, ^{3}J(H,H)=7.7 Hz, 1H, $H_{arom.}$), 7.11 (d, ^{3}J(H,H)=7.8 Hz, 1H, $H_{arom.}$), 4.28 (d, ^{2}J(H,P)=18.8 Hz, 0.29H, PCH), 4.23 (d, ^{2}J(H,P)=18.3 Hz, 0.15H, PCH), 4.14 (d, ^{2}J(H,P)=18.9 Hz, 0.27H, PCH), 4.11 (d, ^{2}J(H,P)=19.0 Hz, 0.12H, PCH), 4.10 (d, ^{2}J(H,P)=19.2 Hz, 0.17H, PCH), 3.39 (dd, ^{2}J(H,H)=14.9 Hz, ^{4}J(H,P)=1.4 Hz, 0.19H, SCH_2), 3.37 (dd, ^{2}J(H,H)=13.4 Hz, ^{4}J(H,P)=1.4 Hz, 0.13H, SCH_2), 3.33-3.32 (m, 0.04H, SCH_2), 3.28 (dd, ^{2}J(H,H)=14.9 Hz, ^{4}J(H,P)=1.6 Hz, 0.31H, SCH_2), 3.19 (dd, ^{2}J(H,H)=15.3 Hz, ^{4}J(H,P)=3.4 Hz, 0.09H, SCH_2), 3.10 (d, ^{2}J(H,H)=15.0 Hz, 0.20H, SCH_2), 3.08 (d, ^{2}J(H,H)=13.2 Hz, 0.10H, SCH_2), 3.06 (dd, ^{2}J(H,H)=13.7 Hz, ^{4}J(H,P)=1.5 Hz, 0.27H, SCH_2), 2.99 (d, ^{2}J(H,H)=14.9 Hz, 0.32H,

SCH_2), 2.87 (d, ^2J(H,H)=14.4 Hz, 0.02H, SCH_2), 2.80 (d, ^2J(H,H)=13.9 Hz, 0.33H, SCH_2), 2.28 ppm (s, 3H, $CH_{3arom.}$); ^{13}C NMR (150.93 MHz, DMSO-d_6): δ=170.9 (C=O), 169.5 (C=O), 169.5 (C=O), 165.9 (C=O), 136.2-136.1 (m, $C_{arom.}$), 136.0 (d, ^5J(C,P)=1.5 Hz, $C_{arom.}$), 134.0 (d, ^2J(C,P)=4.3 Hz, $C_{arom.}$), 133.7 (d, ^2J(C,P)=4.5 Hz, $C_{arom.}$), 133.5 (d, ^2J(C,P)=4.8 Hz, $C_{arom.}$), 129.2-129.0 (m, $C_{arom.}$), 128.7-128.4 (m, $C_{arom.}$), 45.9 (d, ^1J(C,P)=139.5 Hz, PC), 45.9 (d, ^1J(C,P)=139.8 Hz, PC), 45.9 (d, ^1J(C,P)=139.7 Hz, PC), 45.7 (d, ^1J(C,P)=139.7 Hz, PC), 34.6 (d, ^3J(C,P)=5.6 Hz, SCH_2), 33.0 (d, ^3J(C,P)=6.7 Hz, SCH_2), 32.8 (d, ^3J(C,P)=7.2 Hz, SCH_2), 31.4 (d, ^3J(C,P)=6.4 Hz, SCH_2), 20.7-20.5 ppm (m, $CH_{3arom.}$); HPLC analysis: retention time=1.833 min, peak area: 99.83%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.5. ((3,5-Dimethoxyphenyl)((2-(hydroxyamino)-2-oxoethyl)thio)methyl)phosphonic acid **(5h):** light brown oil; yield: 16%. ^{1}H NMR (500.13 MHz, DMSO-d_6): δ=10.75-10.51 (br. s, OH), 10.04 (br. s, OH), 8.94 (br. s, NH), 6.97-6.69 (m, 0.29H, $H_{arom.}$), 6.67-6.48 (m, 1.71H, $H_{arom.}$), 6.47-6.33 (m, 0.85H, $H_{arom.}$), 6.33-6.19 (m, 0.15H, $H_{arom.}$), 4.13-3.95 (m, 1H, PCH), 3.82-3.78 (m, 0.4H, OCH_3), 3.77-3.74 (m, 0.92H, OCH_3), 3.73-3-69 (m, 3.91H, OCH_3), 3.69-3.66 (m, 0.36H, OCH_3), 3.66-3.64 (m, 0.41H, OCH_3), 3.37 (d, ^2J(H,H)=15.0 Hz, 0.48H, SCH_2), 3.29 (d, ^2J(H,H)=14.7 Hz, 0.24H, SCH_2), 3.24 (d, ^2J(H,H)=15.6 Hz, 0.22H, SCH_2), 3.15 (d, ^2J(H,H)=15.0 Hz, 0.35H, SCH_2), 3.07 (d, ^2J(H,H)=13.1 Hz, 0.43H, SCH_2), 2.86 ppm (d, ^2J(H,H)=13.8 Hz, 0.28H, SCH_2); ^{13}C NMR (125.76 MHz, DMSO-d_6): δ=169.5 (C=O), 160.0-159.8 (m, $C_{arom.}$), 139.4-138.8 (m, $C_{arom.}$), 107.9-107.7 (m, $C_{arom.}$), 107.7-107.4 (m, $C_{arom.}$), 98.6-98.5 (m, $C_{arom.}$), 55.1-54.9 (m, OCH_3), 46.4 (d, ^1J(C,P)=138.6 Hz, PC), 46.4 (d, ^1J(C,P)=138.6 Hz, PC), 33.0 (d, ^3J(C,P)=7.5 Hz, SCH_2), 31.5 ppm (d, ^3J(C,P)=7.1 Hz, SCH_2); HPLC analysis: retention time=1.867 min, peak area: 98.94%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.6. ((3,5-Difluorophenyl)((2-(hydroxyamino)-2-oxoethyl)thio)methyl)phosphonic acid **(5i):** brown oil; yield: 82%. ^{1}H NMR (600.22 MHz, DMSO-d_6): δ=11.47 (br. s, OH), 10.57 (br. s, OH), 10.09 (br. s, OH), 8.94 (br. s, NH), 7.21-7.05 (m, 2.5H, $H_{arom.}$), 7.05-6.92 (m, 0.5H, $H_{arom.}$), 4.49 (d, ^2J(H,P)=20.2 Hz, 0.16H, PCH), 4.37 (d, ^2J(H,P)=19.8 Hz, 0.53H, PCH), 4.34-4.28 (m, 0.23H, PCH), 4.25 (d, ^2J(H,P)=19.6 Hz, 0.06H, PCH), 4.25 (d, ^2J(H,P)=19.6 Hz, 0.02H, PCH), 3.99-3.83 (m, 0.34H, SCH_2), 3.73-3.65 (m, 0.08H, SCH_2), 3.59-3.50 (m, 0.09H, SCH_2), 3.47-3.34 (m, 0.13H, SCH_2), 3.29 (d, ^2J(H,H)=15.3 Hz, 0.17H, SCH_2), 3.25 (d, ^2J(H,H)=13.4 Hz, 0.09H, SCH_2), 3.22 (d, ^2J(H,H)=15.7 Hz, 0.02H, SCH_2), 3.19 (d, ^2J(H,H)=14.7 Hz, 0.04H, SCH_2),

3.12 (d, ^{2}J(H,H)=15.1 Hz, 0.13H, SCH_2), 3.04 (d, ^{2}J(H,H)=13.4 Hz, 0.16H, SCH_2), 3.03 (d, ^{2}J(H,H)=13.9 Hz, 0.37H, SCH_2), 2.89 (d, ^{2}J(H,H)=13.9 Hz, 0.11H, SCH_2), 2.85 ppm (d, ^{2}J(H,H)=13.9 Hz, 0.27H, SCH_2); ^{13}C NMR (150.93 MHz, DMSO-d_6): δ=165.3 (C=O), 161.8 (dd, ^{1}J(C,F)=245.4 Hz, ^{3}J(C,F)=12.3 Hz, $C_{arom.}$), 142.3-141.7 (m, $C_{arom.}$), 134.4-134.1 (m, $C_{arom.}$), 112.3 (d, ^{2}J(C,F)=20.3 Hz, $C_{arom.}$), 102.9-102.1 (m $C_{arom.}$), 45.9 (d, ^{1}J(C,P)=137.3 Hz, PC), 31.5 ppm (d, ^{3}J(C,P)=7.6 Hz, SCH_2); HPLC analysis: retention time=1.883 min, peak area: 97.15%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.7. (((2-(Hydroxy(methyl)amino)-2-oxoethyl)thio)(4-(methylthio)phenyl)methyl)phosphonic acid **(5j):** white solid; yield: 80%. M.p. 134 °C; ^{1}H NMR (600.22 MHz, DMSO-d6): δ=10.00 (br. s, OH), 7.33 (dd, ^{3}J(H,H)=8.3 Hz, ^{4}J(H,H)=1.6 Hz, 2H, $H_{arom.}$), 7.20 (d, ^{3}J(H,H)=8.3 Hz, 2H, $H_{arom.}$), 4.23 (d, ^{2}J(H,P)=19.2 Hz, 1H, PCH), 3.48 (d, ^{2}J(H,H)=14.6 Hz, 1H, SCH_2), 3.21 (d, ^{2}J(H,H)=14.6 Hz, 1H, SCH_2), 3.06 (s, 3H, NCH_3), 2.46 ppm (s, 3H, SCH_3); ^{13}C NMR (150.93 MHz, DMSO-d_6): δ=168.9 (C=O), 136.5 ($C_{arom.}$), 133.9 (d, ^{2}J(C,P)=4.1 Hz, $C_{arom.}$), 129.8 (d, ^{3}J(C,P)=5.8 Hz, $C_{arom.}$), 125.5 ($C_{arom.}$), 45.3 (d, ^{1}J(C,P)=139.5 Hz, PC), 35.7 (NCH_3), 31.8 (d, ^{3}J(C,P)=6.5 Hz, SCH_2), 14.6 ppm (SCH_3); HPLC analysis: retention time=2.011 min, peak area: 98.90%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 30 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.8. (((2-(Hydroxy(methyl)amino)-2-oxoethyl)sulfonyl)(phenyl)methyl)phosphonic acid **(7a):** colorless oil; yield: 26%. ^{1}H NMR (500.13 MHz, DMSO-d_6): δ=10.37 (br. s, OH), 7.55-7.45 (m, 2H, $H_{arom.}$), 7.43-7.30 (m, 3H, $H_{arom.}$), 5.50 (d, ^{2}J(H,P)=19.1 Hz, 0.9H, PCH), 5.42 (d, ^{2}J(H,P)=19.1 Hz, 0.1H, PCH), 4.95 (d, ^{2}J(H,H)=15.2 Hz, 0.92H, SCH_2), 4.72 (d, ^{2}J(H,H)=15.0 Hz, 0.08H, SCH_2), 4.55 (d, ^{2}J(H,H)=15.2 Hz, 0.08H, SCH_2), 4.34 (d, ^{2}J(H,H)=15.2 Hz, 0.92H, SCH_2), 3.19 (s, 0.15H, NCH_3), 3.15 (s, 2.69H, NCH_3), 3.11 ppm (s, 0.16H, NCH_3); ^{13}C NMR (125.76 MHz, DMSO-d_6): δ=162.2 (C=O), 131.2 (d, ^{2}J(C,P)=5.2 Hz, $C_{arom.}$), 128.8 (d, ^{3}J(C,P)=5.2 Hz, $C_{arom.}$), 128.2 ($C_{arom.}$), 128.2 ($C_{arom.}$), 67.2 (d, ^{1}J(C,P)=129.4 Hz, PC), 54.0 (SCH_2), 35.6 ppm (NCH_3); HPLC analysis: retention time=1.790 min, peak area: 99.16%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.9. (((2-(Hydroxy(methyl)amino)-2-oxoethyl)sulfonyl)(p-tolyl)methyl)phosphonic acid **(7b):** brown oil; yield: 61%. ^{1}H NMR (600.22 MHz, DMSO-d_6): δ=10.36 (br. s, OH), 7.37 (d,

^{3}J(H,H)=7.3 Hz, 2H, $H_{arom.}$), 7.18 (d, ^{3}J(H,H)=7.9 Hz, 2H, $H_{arom.}$), 5.4 (d, ^{2}J(H,P)=19.1 Hz, 0.8H, PCH), 5.35 (d, ^{2}J(H,P)=18.9 Hz, 0.06H, PCH), 5.13 (d, ^{2}J(H,P)=19.0 Hz, 0.06H, PCH), 5.12 (d, ^{2}J(H,P)=19.0 Hz, 0.08H, PCH), 4.92 (d, ^{2}J(H,H)=15.1 Hz, 0.78H, SCH_2), 4.77 (d, ^{2}J(H,H)=14.5 Hz, 0.04H, SCH_2), 4.76 (d, ^{2}J(H,H)=14.5 Hz, 0.04H, SCH_2), 4.70 (d, ^{2}J(H,H)=14.6 Hz, 0.14H, SCH_2), 4.52 (d, ^{2}J(H,H)=15.1 Hz, 0.06H, SCH_2), 4.48 (d, ^{2}J(H,H)=14.4 Hz, 0.04H, SCH_2), 4.44 (d, ^{2}J(H,H)=14.5 Hz, 0.04H, SCH_2), 4.31 (d, ^{2}J(H,H)=15.1 Hz, 0.77H, SCH_2), 4.24 (d, ^{2}J(H,H)=14.6 Hz, 0.09H, SCH_2), 3.14 (s, 3H, NCH_3), 2.31 ppm (s, 3H, $CH_{3arom.}$); ^{13}C NMR (150.93 MHz, DMSO-d_6): δ=162.3 (C=O), 137.7 ($C_{arom.}$), 131.1 (d, ^{2}J(C,P)=5.4 Hz, $C_{arom.}$), 128.8 ($C_{arom.}$), 125.7 (d, ^{3}J(C,P)=5.3 Hz, $C_{arom.}$), 66.9 (d, ^{1}J(C,P)=130.1 Hz, PC), 53.9 (SCH_2), 35.5 (NCH_3), 20.7 ppm ($CH_{3arom.}$); HPLC analysis: retention time=1.870 min, peak area: 99.91%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.10. (((2-(Hydroxy(methyl)amino)-2-oxoethyl)sulfonyl)(naphthalen-1-yl)methyl) phosphonic acid **(7c):** beige-colored oil; yield: 34%. ^{1}H NMR (600.22 MHz, DMSO-d_6): δ=10.46 (br. s, OH), 8.23 (d, ^{3}J(H,H)=8.6 Hz, 0.8H, $H_{arom.}$), 8.19-8.14 (m, 0.2H, $H_{arom.}$), 8.08 (d, ^{3}J(H,H)=7.2 Hz, 1H, $H_{arom.}$), 8.01-7.94 (m, 2H, $H_{arom.}$), 7.65-7.59 (m, 1H, $H_{arom.}$), 7.58-7.52 (m, 2H, $H_{arom.}$), 6.65 (d, ^{2}J(H,P)=20.3 Hz, 0.79H, PCH), 6.55 (d, ^{2}J(H,P)=21.0 Hz, 0.05H, PCH), 6.27 (d, ^{2}J(H,P)=20.1 Hz, 0.07H, PCH), 6.18 (d, ^{2}J(H,P)=20.1 Hz, 0.09H, PCH), 5.29 (d, ^{2}J(H,H)=15.3 Hz, 0.78H, SCH_2), 5.23 (d, ^{2}J(H,H)=14.8 Hz, 0.1H, SCH_2), 5.17 (d, ^{2}J(H,H)=14.9 Hz, 0.12H, SCH_2), 4.84 (d, ^{2}J(H,H)=15.0 Hz, 0.04H, SCH_2), 4.73 (d, ^{2}J(H,H)=15.0 Hz, 0.07H, SCH_2), 4.60 (d, ^{2}J(H,H)=14.8 Hz, 0.09H, SCH_2), 4.51 (d, ^{2}J(H,H)=15.4 Hz, 0.71H, SCH_2), 4.38 (d, ^{2}J(H,H)=14.8 Hz, 0.09H, SCH_2), 3.34-3.29 (m, 0.16H, NCH_3), 3.19 ppm (s, 2.84H, NCH_3); ^{13}C NMR (150.93 MHz, DMSO-d_6): δ=162.8 (C=O), 133.5 ($C_{arom.}$), 132.7 (d, J(C,P)=6.2 Hz, $C_{arom.}$), 129.0 (d, J(C,P)=4.3 Hz, $C_{arom.}$), 128.8 ($C_{arom.}$), 128.6 ($C_{arom.}$), 126.6 ($C_{arom.}$), 125.6 ($C_{arom.}$), 125.0-124.9 (m, $C_{arom.}$), 124.3 (d, J(C,P)=5.0 Hz, $C_{arom.}$), 123.4 ($C_{arom.}$), 60.8 (d, ^{1}J(C,P)=131.2 Hz, PC), 54.5 (SCH_2), 35.7 (NCH_3), 34.6 ppm (NCH_3); HPLC analysis: retention time=1.877 min, peak area: 99.95%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.11. ((3,4-Dichlorophenyl)((2-(hydroxy(methyl)amino)-2-oxoethyl)sulfonyl)methyl) phosphonic acid **(7d):** brown oil; yield: 17%. ^{1}H NMR (600.22 MHz, DMSO-d_6): δ=10.47 (br. s, OH), 7.79-7.76 (m, 0.03H, $H_{arom.}$), 7.76-7.75 (m, 0.03H, $H_{arom.}$), 7.75-7.71 (m, 0.94H, $H_{arom.}$), 7.68 (d, ^{3}J(H,H)=8.4 Hz, 1H, $H_{arom.}$), 7.46 (d, ^{3}J(H,H)=8.3 Hz, 1H, $H_{arom.}$), 5.50 (d, ^{2}J(H,P)=18.7 Hz,

0.95H, PCH), 5.28 (d, ^{2}J(H,P)=18.4 Hz, 0.02H, PCH), 5.21 (d, ^{2}J(H,P)=18.3 Hz, 0.03H, PCH), 4.90 (d, ^{2}J(H,H)=15.2 Hz, 1H, SCH_2), 4.42 (d, ^{2}J(H,H)=15.2 Hz, 0.9H, SCH_2), 4.37 (d, ^{2}J(H,H)=14.5 Hz, 0.07H, SCH_2), 4.30 (d, ^{2}J(H,H)=15.4 Hz, 0.03H, SCH_2), 3.15 ppm (s, NCH_3); ^{13}C NMR (150.93 MHz, DMSO- d_6): δ=161.9 (C=O), 132.5 (d, ^{2}J(C,P)=4.7 Hz, $C_{arom.}$), 131.6 (d, ^{3}J(C,P)=4.8 Hz, $C_{arom.}$), 131.3 ($C_{arom.}$), 130.7 ($C_{arom.}$), 130.4 ($C_{arom.}$), 130.1 (d, ^{3}J(C,P)=4.9 Hz, $C_{arom.}$), 66.1 (d, ^{1}J(C,P)=126.6 Hz, PC), 54.2 (SCH_2), 35.5 ppm (NCH_3); HPLC analysis: retention time=1.817 min, peak area: 99.02%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.12. ((3,5-Difluorophenyl)((2-(hydroxy(methyl)amino)-2-oxoethyl)sulfonyl)methyl) phosphonic acid **(7e):** brown oil; yield: 80%. ^{1}H NMR (600.22 MHz, DMSO- d_6): δ=10.48 (br. s, NOH), 7.33-7.27 (m, 1H, $H_{arom.}$), 7.23 (d, ^{3}J(H,F)=7.6 Hz, 0.45H, $H_{arom.}$), 7.19 (d, ^{3}J(H,F)=7.1 Hz, 1.55H, $H_{arom.}$), 5.51 (d, ^{2}J(H,P)=18.6 Hz, 0.71H, PCH), 5.39 (d, ^{2}J(H,P)=18.1 Hz, 0.07H, PCH), 5.29 (d, ^{2}J(H,P)=18.5 Hz, 0.06H, PCH), 5.22 (d, ^{2}J(H,P)=17.5 Hz, 0.16H, PCH), 4.97 (d, ^{2}J(H,H)=15.0 Hz, 0.07H, SCH_2), 4.88 (d, ^{2}J(H,H)=15.1 Hz, 0.58H, SCH_2), 4.71 (d, ^{2}J(H,H)=14.1 Hz, 0.06H, SCH_2), 4.63 (d, ^{2}J(H,H)=14.4 Hiiz, 0.23H, SCH_2), 4.57 (d, ^{2}J(H,H)=14.5 Hz, 0.06H, SCH_2), 4.42 (d, ^{2}J(H,H)=15.1 Hz, 0.78H, SCH_2), 4.37 (d, ^{2}J(H,H)=14.4 Hz, 0.22H, SCH_2), 3.74 (br. s, POH), 3.15 ppm (NCH_3); ^{13}C NMR (150.93 MHz, DMSO- d_6): δ=161.9 (C=O), 161.7 (dd, ^{1}J(C,F)=245.5 Hz, ^{3}J(C,F)=12.9 Hz, $C_{arom.}$), 133.1-133.0 (m, $C_{arom.}$), 114.3 (dd, ^{2}J(C,F)=21.0 Hz, ^{4}J(C,F)=^{3}J(C,P)=5.2 Hz, $C_{arom.}$), 104.2-103.8 (m, $C_{arom.}$), 66.5 (d, ^{1}J(C,P)=127.4 Hz, PC), 54.2 (SCH_2), 35.6 ppm (NCH_3); HPLC analysis: retention time=1.780 min, peak area: 99.51%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.13. ((3,5-Dimethoxyphenyl)((2-(hydroxy(methyl)amino)-2-oxoethyl)sulfonyl)methyl) phosphonic acid **(7f):** beige-colored oil; yield: 41%. ^{1}H NMR (600.22 MHz, DMSO- d_6): δ=10.54 (br. s, NOH), 6.76-6.72 (m, 0.2H, $H_{arom.}$), 6.72-6.62 (m, 1.8H, $H_{arom.}$), 6.55-6.46 (m, 1H, $H_{arom.}$), 5.33 (d, ^{2}J(H,P)=18.9 Hz, 0.61H, PCH), 5.21 (d, ^{2}J(H,P)=18.3 Hz, 0.39H, PCH), 5.00 (d, ^{2}J(H,H)=15.2 Hz, 0.28H, SCH_2), 4.91 (d, ^{2}J(H,H)=15.1 Hz, 0.5H, SCH_2), 4.64 (d, ^{2}J(H,H)=14.4 Hz, 0.22H, SCH_2), 4.55 (d, ^{2}J(H,H)=14.8 Hz, 0.17H, SCH_2), 4.32 (d, ^{2}J(H,H)=15.1 Hz, 0.47H, SCH_2), 4.31 (d, ^{2}J(H,H)=14.6 Hz, 0.19H, SCH_2), 4.23 (d, ^{2}J(H,H)=13.9 Hz, 0.17H, SCH_2), 3.76-3.70 (m, 6H, OCH_3), 3.65 (br. s, POH), 3.17-3.12 ppm (m, 3H, NCH_3); ^{13}C NMR (150.93 MHz, DMSO- d_6): δ=162.2 (C=O), 159.8 ($C_{arom.}$), 159.7 ($C_{arom.}$), 109.6 (d, ^{3}J(C,P)=3.1 Hz, $C_{arom.}$), 99.60 ($C_{arom.}$), 55.1-55.0 (m, OCH_3), 54.1-54.0 (m, SCH_2), 35.5 ppm (NCH_3); HPLC analysis: retention

time=1.853 min, peak area: 99.72%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.1.14. (((2-(Hydroxyamino)-2-oxoethyl)sulfonyl)(p-tolyl)methyl)phosphonic acid **(7g):** brown oil; yield: 92%. ^{1}H NMR (600.22 MHz, DMSO- d_6): δ=10.88 (br. s, OH), 10.54 (br. s, OH), 10.10 (br. s, OH), 9.33 (br. s, NH), 7.42 (d, ^3J(H,H)=7.7 Hz, 0.19H, $H_{arom.}$), 7.40-7.38 (m, 0.25H, $H_{arom.}$), 7.36 (d, ^3J(H,H)=7.7 Hz, 1.56H, $H_{arom.}$), 7.22-7.20 (m, 0.12H, $H_{arom.}$), 7.18 (d, ^3J(H,H)=7.8 Hz, 1.88H, $H_{arom.}$), 5.40 (d, ^2J(H,P)=19.3 Hz, 0.7H, PCH), 5.27 (d, ^2J(H,P)=19.0 Hz, 0.04H, PCH), 5.25 (d, ^2J(H,P)=18.6 Hz, 0.04H, PCH), 5.21 (d, ^2J(H,P)=18.9 Hz, 0.02H, PCH), 5.16 (d, ^2J(H,P)=19.0 Hz, 0.12H, PCH), 5.12 (d, ^2J(H,P)=21.9 Hz, 0.04H, PCH), 5.09 (d, ^2J(H,P)=22.1 Hz, 0.04H, PCH), 4.84 (d, ^2J(H,H)=15.1 Hz, 0.05H, SCH_2), 4.80 (d, ^2J(H,H)=14.2 Hz, 0.11H, SCH_2), 4.70 (d, ^2J(H,H)=14.6 Hz, 0.11H, SCH_2), 4.59 (d, ^2J(H,H)=14.2 Hz, 0.7H, SCH_2), 4.48 (d, ^2J(H,H)=14.3 Hz, 0.02H, SCH_2), 4.44 (d, ^2J(H,H)=14.5 Hz, 0.01H, SCH_2), 4.25 (d, ^2J(H,H)=14.7 Hz, 0.12H, SCH_2), 4.21 (d, ^2J(H,H)=15.2 Hz, 0.05H, SCH_2), 4.09 (d, ^2J(H,H)=14.4 Hz, 0.1H, SCH_2), 3.74 (d, ^2J(H,H)=13.2 Hz, 0.73H, SCH_2), 2.30 ppm (s, 3H, $CH_{3arom.}$); ^{13}C NMR (150.93 MHz, DMSO- d_6): δ=158.9 (C=O), 137.6 ($C_{arom.}$), 131.14-130.9 (m, $C_{arom.}$), 128.8 ($C_{arom.}$), 125.8 (d, ^3J(C,P)=5.3 Hz, $C_{arom.}$), 66.8 (d, ^1J(C,P)=130.2 Hz, PC), 55.2 (SCH_2), 20.7 ppm ($CH_{3arom.}$); HPLC analysis: retention time=1.833 min, peak area: 99.75%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm

5.1.1.15. ((3,5-Dimethoxyphenyl)((2-(hydroxyamino)-2-oxoethyl)sulfonyl)methyl)phosphonic acid **(7h):** white solid; yield: 80%. M.p. 167 °C; ^{1}H NMR (600.22 MHz, DMSO- d_6): δ=10.89 (br. s, NOH), 10.55 (br. s, NOH), 10.09 (br. s, NOH), 9.58 (br. s, NH), 9.35 (br. s, NH), 6.93-6.91 (m, 0.05H, $H_{arom.}$), 6.80-6.75 (m, 0.07H, $H_{arom.}$), 6.73-6.72 (m, 0.03H, $H_{arom.}$), 6.72-6.70 (m, 0.05H, $H_{arom.}$), 6.65 (s, 1.8H, $H_{arom.}$), 6.59-6.56 (m, 0.11H, $H_{arom.}$), 6.55-6.50 (m, 0.84H, $H_{arom.}$), 6.41-6.38 (m, 0.05H, $H_{arom.}$), 6.13 (d, ^2J(H,P)=19.3 Hz, 0.02H, PCH), 5.82 (d, ^2J(H,P)=19.2 Hz, 0.02H, PCH), 5.35 (d, ^2J(H,P)=19.3 Hz, 0.77H, PCH), 5.26 (d, ^2J(H,P)=19.6 Hz, 0.07H, PCH), 5.13 (d, ^2J(H,P)=19.1 Hz, 0.12H, PCH), 5.02 (d, ^2J(H,H)=14.2 Hz, 0.02H, SCH_2), 4.80 (d, ^2J(H,H)=15.0 Hz, 0.05H, SCH_2), 4.77 (d, ^2J(H,H)=13.9 Hz, 0.04H, SCH_2), 4.64 (d, ^2J(H,H)=14.6 Hz, 0.09H, SCH_2), 4.53 (d, ^2J(H,H)=14.2 Hz, 0.33H, SCH_2), 4.44 (d, ^2J(H,H)=14.2 Hz, 0.19H, SCH_2), 4.39 (br. s, POH), 4.28 (d, ^2J(H,H)=14.6 Hz, 0.14H, SCH_2), 4.23 (d, ^2J(H,H)=16.2 Hz, 0.13H, SCH_2), 3.78 (d, ^2J(H,H)=14.2 Hz, 1H, SCH_2), 3.74 (s, 5.6H, OCH_3), 3.72 ppm (s, 0.4H, OCH_3); ^{13}C NMR (150.93 MHz, DMSO- d_6): δ=159.9 (C=O), 158.7 ($C_{arom.}$), 130.7 (d, ^2J(C,P)=5.2

Hz, $C_{arom.}$), 109.5 (d, ^{3}J(C,P)=4.9 Hz, $C_{arom.}$), 99.7 ($C_{arom.}$), 67.0 (d, ^{1}J(C,P)=128.7 Hz, PC), 55.3-54.9 ppm (m, OCH_3, SCH_2); HPLC analysis: retention time=1.833 min, peak area: 99.79%, eluent A: NH_4OAc solution (5 mM), eluent B: CH_3CN, isocratic (1:1) over 20 min with a flow rate of 1 mL min^{-1} and detection at 254 nm.

5.1.2. Chiral separation of compound 5a

Chiral separation of compound **5a** was performed with a Chiracel OZ-H column (5µM particle size, 250 mm x 4.6 mm, Chiral Technologies, France) on an HP series 1100 HPLC (Agilent Technologies). After injection of 50 µL of 35 mM **5a** in n-heptane / propan-2-ol (70:30) at a flow rate of 1 mL min^{-1}, the column was developed at the same flow rate at 25 °C with n-heptane / propan-2-ol / (70:30) containing 0.075% trifluoroacetic acid. 1 mL fractions were collected after 5 min (*R)*-**5a** = inactive enantiomer) and after 7 min ((*S*)-**5a** = active enantiomer). Sodium phosphate (pH 7.4) was added to a final concentration of 25 mM. The organic phase was discarded. The aqueous phase was concentrated to 3 mM enantiomer with a nitrogen stream. A Nucleosil 100-5 C18 HD column (250 x 10 mm, Machery-Nagel) on a 1260 Infinity Preparative Scale HPLC (Agilent Technologies) was used to separate each enantiomer from Na-phosphate and trifluoroacetic acid. After injection of 5 mL enantiomer containing solution, the column was developed with water at a flow rate of 1 mL min^{-1} at 25 °C. Fractions were collected and lyophilized at -30 °C and subsequently stored at -80 °C.

*5.1.3. Stability of **5a** in the 100 mM Tris-HCl, pH 7.6.*

Compound **5a** (racemate, (*R*)-**5a** or (*S*)-**5a**, with concentrations between 0.6 mM and 0.7 mM) were incubated at room temperature (22 ^{o}C – 23 ^{o}C) for two days in the 100 mM Tris-HCl pH 7.6, 0.02% NaN_3. At time points of 0 hours, 24 hours and 48 hours of incubation aliquots were taken away from each sample and stored at -80 ^{o}C. All collected aliquots were tested for IC_{50} value in parallel in the photometric assay as described in the previous paragraph.

*5.1.4. Stability of compound **5a**. Sample preparation and stability measurement.*

1.0 mg of **5a** were dissolved with phosphate buffer pH 7.6 (prepared according to Ph. Eur. 5.0) to obtain a final concentration of 1 mg/mL. The resulting solution was shaken on an IKA KS 260 basic shaker at room temperature for 72 hours. 10 µl of this solution were used for HPLC analysis at each time point (0, 1, 3, 6 24, 72h, n = 3). Decomposition was determined by the relative reduction of the area under the curve (AUC) at 254 nm.[41] For stability testing a Knauer HPLC with a Knauer UV detector K-2600 (254nm) was used to identify decomposition. Column type Vertex Plus Column (length 150 x 4 mm with precolumn, packing material of the column was Eurospher II 100-5 C18) was used with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (10 to 100 %) in water containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min over 30 min, isocratic elution of acetonitrile containing 0.1% trifluoroacetic acid (100% over 10 min. Retention time of **5a**: 4.1 min.

5.2. **Biological evaluation:**

5.2.1.1. IspC enzyme Assays

5.2.1.1.1. *Gene expression and protein purification*

The *isp*C of *P. falciparum* was expressed in *E. coli* and the protein was purified as described earlier.[20] IspC orthologs of *E. coli* or *M. tuberculosis* were isolated and purified as follows. The recombinant *E. coli* strain M15 pREP4 containing pQE*isp*C-Ec (for the *E. coli* enzyme) or pQE*isp*C-Mt (for the *M. tuberculosis* enzyme) was grown to an optical density of 0.4-0.6 at 37 °C in LB medium supplemented with ampicillin (70 mg L^{-1}) and kanamycin (30 mg L^{-1}). Isopropylthiogalactoside was added to a final concentration of 1 mM, and the cell suspension was cultivated 36 hours at 20 °C under shaking (140 rpm). Cells were harvested by centrifugation, washed with 0.9% NaCl solution, and resuspended in loading buffer (50 mM Tris hydrochloride, pH 8.0, containing 0.5 M NaCl, 20 mM imidazole and 0.02% NaN_3). The suspension was passed through a cell disruption device (FrenchPress, Constant Cell Disruption Systems, Koenigswinter, Germany) and was then centrifuged (15 000 rpm, +4 ^{o}C, 1h). The supernatant was subjected to a Ni^{2+}-chelating Sepharose fast flow column (volume

of 30 mL) that had been pre-equilibrated with loading buffer. The column was washed with 150 mL of the loading buffer and was then developed with imidazole gradient (20 mM – 500 mM) in laoding buffer. Fractions of eluent were combined and dialyzed overnight against 50 mM Tris hydrochloride, pH 8.0, containing 2 mM DTT and 0.02% NaN_3. IspC batches were obtained with approximately 95% purity, as estimated by SDS−PAGE analysis.

5.2.1.2. Photometric Assay

Assays were conducted in 384-well plates with flat clear bottom (Nunc, Wiesbaden, Germany). Assay mixtures with a total volume of 60 µL contained 100 mM Tris hydrochloride, pH 7.6, 5 mM $MnCl_2$, 5 mM DTT, 0.5 mM NADPH, 0.02 U of IspC protein, and test compounds. Dilution series (1:3) of test compounds covered the concentration range of 200 - 0.003 µM. The reaction was started by addition of 1-deoxy-D-xylulose 5-phosphate in 100 mM Tris hydrochloride, pH 7.6, to a final concentration of 0.5 mM. The reaction was monitored photometrically (room temperature) at 340 nm using a plate reader (SpectraMax M5, Molecular Devices, Biberach an der Riss, Germany). Inhibition constant (*K*i) and IC_{50} values were calculated with the program Dynafit [35] as described earlier.[18]

5.2.2. In vitro antiplasmodial activity.

Acitivity against asexual blood stages was evaluated based on a histidine rich protein 2 (HRP2) enzyme linked immunosorbent assay (ELISA) as reported previously.[42,43] The *in vitro* toxicity was tested via a spectrophotometric quantification of cytotoxicity against HeLa cells.[44]

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Author Contributions

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Appendix A. Supplementary data

Experimental procedures, analytical data for compounds **3e**-**f**, **4a**-**j** and **6a**-**h,** Computational Study of (*S)*-**5a** and (*S)*-**7a** and antibacterial activity are provided as supplementary information.

Abbreviations

ABS, asexual blood stage; DMAPP, Dimethylallyl pyrophosphate, DOXP, 1-deoxy-D-xylulose 5-phosphate, DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *Ec*IspC, *Escherichia coli* IspC enzyme; HeLa, Henrietta Lacks; HRP2, histidine rich protein IPP, isopentenyl pyrophosphate mp, melting point; MSD, mean survival time in days; MEP, 2-C-methyl-D-erythritol 4-phosphate; *Mc*IspC, *Mycobacterium tuberculosis* IspC enzyme NMRI, Naval Medical Research Institute *Pf*3D7, *Plasmodium* strain 3D7; *Pf*Dd2, *Plasmodium* strain Dd2; *Pf*IspC, *P. falciparum* IspC enzyme; SI, selectivity index; SD, Standard deviation TMSBr, Bromotrimethylsilane; UHR-TOF, ultrahigh resolution – time of flight

Conflicts of interest

The authors declare that they have no conflict of interest.

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Highlights

- Synthesis of novel α-phenyl-substituted β-thia-isosteric fosmidomycin analogs.
- The new inhibitors exert nanomolar activity against *P. falciparum* and *E. coli* IspC.
- Compounds **5e-j** showed potent antiplasmodial activity against *P. falciparum.*
- The **5a** (*S*)-(+)-enantiomer showed higher antiplasmodial activity than the racemate.