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Inhibition of DXR in the MEP pathway with lipophilic *N*-alkoxyaryl FR900098 analogs†

Darean Bague,^{‡a} Ruiqin Wang,^{‡a} Dana Hodge,^{id d} Marwa O. Mikati,^{id e} Jose S. Roma,^c Helena I. Boshoff,^{id c} Allyson L. Dailey,^b Misgina Girma,^b Robin D. Couch,^{id b} Audrey R. Odom John^{id de} and Cynthia S. Dowd^{id *a}

In *Mycobacterium tuberculosis* (*Mtb*) and *Plasmodium falciparum* (*Pf*), the methylerythritol phosphate (MEP) pathway is responsible for isoprene synthesis. This pathway and its products are vital to bacterial/parasitic metabolism and survival, and represent an attractive set of drug targets due to their essentiality in these pathogens but absence in humans. The second step in the MEP pathway is the conversion of 1-deoxy-D-xylulose-5-phosphate (DXP) to MEP and is catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR). Natural products fosmidomycin and FR900098 inhibit DXR, but are too polar to reach the desired target inside some cells, such as *Mtb*. Synthesized FR900098 analogs with lipophilic substitution in the position α to the phosphorous atom showed promise, resulting in increased activity against *Mtb* and *Pf*. Here, an α substitution, consisting of a 3,4-dichlorophenyl substituent, in combination with various *O*-linked alkylaryl substituents on the hydroxamate moiety is utilized in the synthesis of a novel series of FR900098 analogs. The purpose of the *O*-linked alkylaryl substituents is to further enhance DXR inhibition by extending the structure into the adjacent NADPH binding pocket, blocking the binding of both DXP and NADPH. Of the initial *O*-linked alkylaryl substituted analogs, compound **6e** showed most potent activity against *Pf* parasites at 3.60 μ M. Additional compounds varying the phenyl ring of **6e** were synthesized. The most potent phosphonic acids, **6l** and **6n**, display nM activity against *Pf*DXR and low μ M activity against *Pf* parasites. Prodrugs of these compounds were less effective against *Pf* parasites but showed modest activity against *Mtb* cells. Data from this series of compounds suggests that this combination of substituents can be advantageous in designing a new generation of antimicrobials.

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Introduction

Tuberculosis (TB) and malaria still pose a significant threat to global public health despite important research efforts toward eradication. The major causative agents of TB and malaria are *Mycobacterium tuberculosis* (*Mtb*) and *Plasmodium falciparum* (*Pf*), respectively. The World Health Organization (WHO) reported an estimated combined 2.2 million deaths related to TB and malaria globally in 2021.^{1,2}

Further exacerbating the situation, there has been a rapid increase in *Mtb* and *Pf* drug resistance, leaving limited therapeutic options.^{3–10} The WHO reported 1.5 million cases of drug-resistant TB in 2021.² Similarly, in regions with a high malaria burden, such as the Greater Mekong Subregion (GMS) and Africa, resistance to artemisinin-combination therapy (ACT) is rising at an alarming rate.^{1,5–7,10} This threat to global public health requires the discovery and validation of novel drug targets that are not currently being used by existing antibiotics.

The 2C-methyl-D-erythritol-4-phosphate (MEP, or nonmevalonate) pathway for isoprene synthesis (Fig. 1) is found in several pathogens, including *Mtb* and *Pf*.^{11–16} Isoprene isomers isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are vital as they are converted to isoprenoids which are essential to cell wall synthesis and other cellular functions.^{17–20} Although isoprene synthesis is also vital to humans, we synthesize isoprenes through an alternate pathway. This makes the MEP pathway an attractive set of drug targets. Interrupting synthesis of isoprenes is lethal for *Mtb* and *Pf* cells, thus providing a novel

^a Department of Chemistry, George Washington University, Washington, D.C. 20052, USA. E-mail: cdowd@gwu.edu

^b Department of Chemistry and Biochemistry, George Mason University, Fairfax, VA 22030, USA

^c Tuberculosis Research Section, LCIM, NIAID/NIH, Bethesda, MD 20892, USA

^d Division of Infectious Diseases, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

^e Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, 63110, USA

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‡ These authors contributed equally to this work.



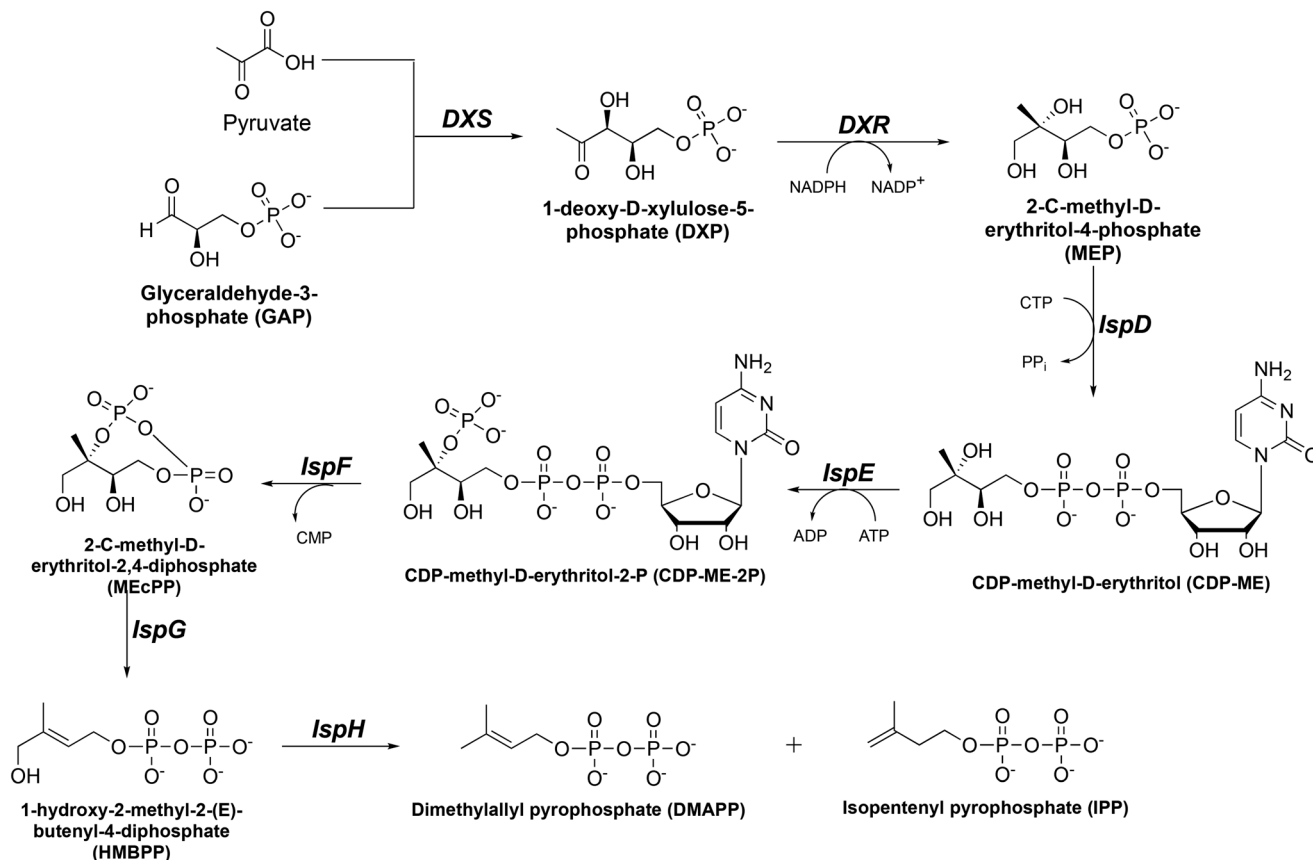


Fig. 1 The methyl erythritol phosphate (MEP) pathway of isoprene synthesis.

method to combat both drug-sensitive and drug-resistant strains of *Mtb* and *Pf*.

In the MEP pathway, 1-deoxy-D-xylulose-5-phosphate (DXP) is isomerized and reduced to MEP (Fig. 1).^{14–20} This step is catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), which is a validated drug target for malaria.^{21–24} Fosmidomycin (Fos, **1**) and FR900098 (**2**) are natural products produced by *Streptomyces lavendulae* and *Streptomyces*

rubellomurinus, respectively, which have antimicrobial activities against Gram-negative bacteria (Fig. 2).^{25–32} These compounds are potent inhibitors of DXR, mimic the DXP substrate, and bind to DXR at the DXP binding site. Although potent, Fos and FR900098 are very polar and can display poor cellular activity.^{16–28} Thus, they have been frequent starting points for inhibitor design. Fos and FR900098 have four positions where modifications could enhance *Mtb* and *Pf*

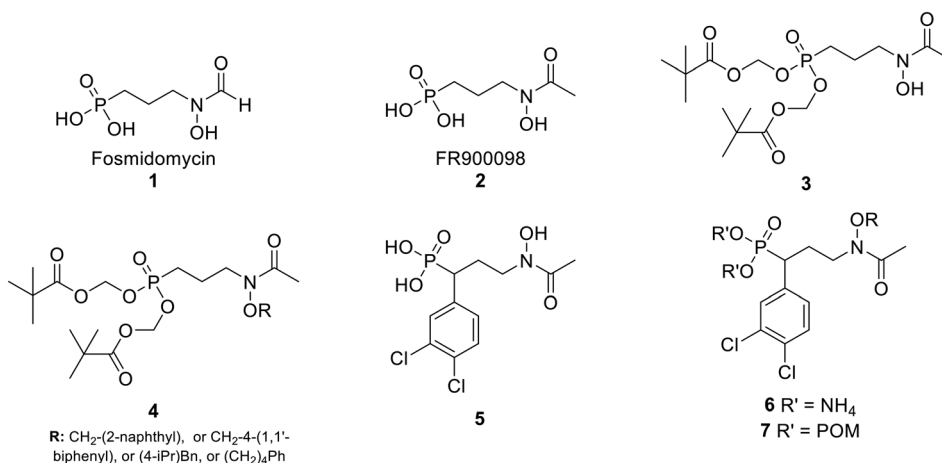


Fig. 2 Fosmidomycin (**1**), FR900098 (**2**), previously reported compounds (**3–5**), and compounds in current work (**6–7**).

activity: the phosphonate, the propyl backbone, the *N*-hydroxyl moiety, and the *N*-acyl substituent. We and others have synthesized analogs in which all of these moieties were modified, and the resulting analogs were tested for activity against *Mtb* and *Pf*.^{33–53} Additionally, these compounds (for example, **3** and **4**) often have better cell permeability due to increased lipophilicity.^{29,54}

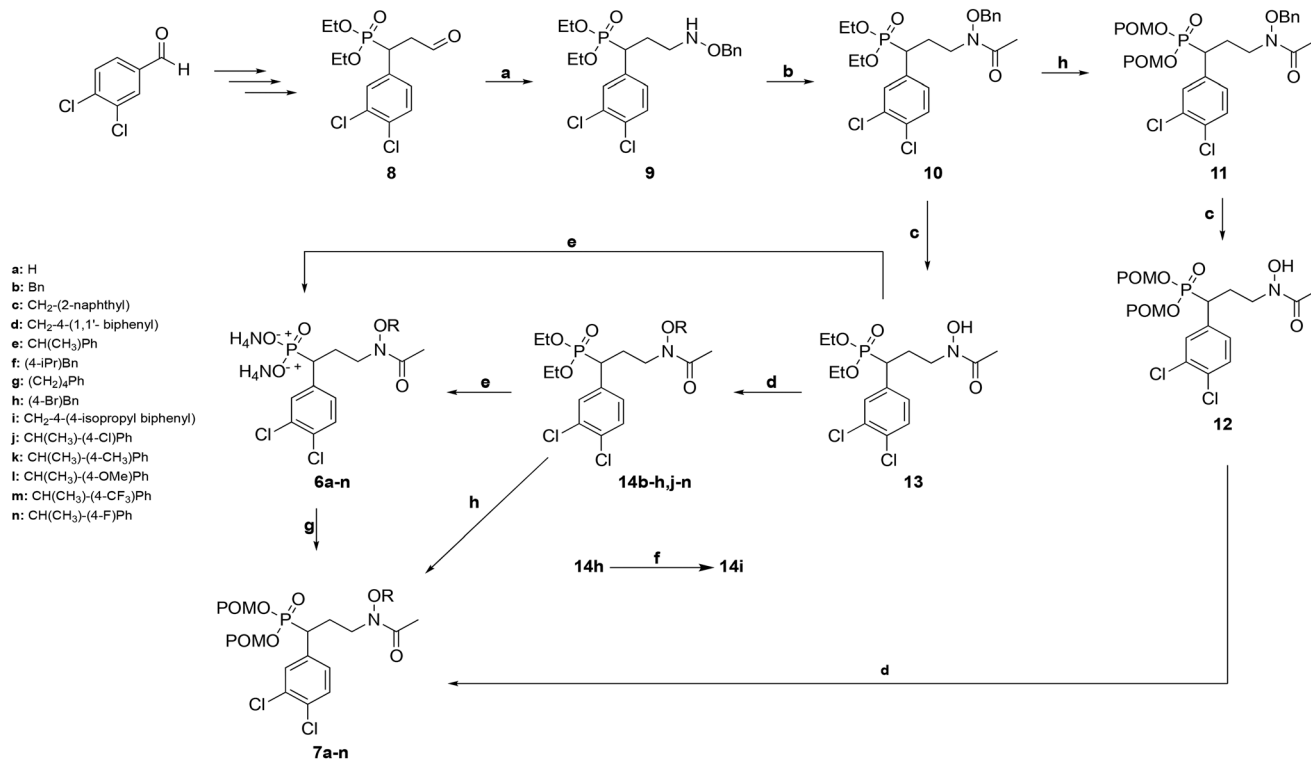
In the current work, we combine two structural features, previously shown to be advantageous to improving antimicrobial activity, in an effort to design a novel family of MEP pathway inhibitors, termed MEPicides. In 2006, the Van Calenbergh group reported a series of analogs bearing an aryl substituent alpha to the phosphonate moiety.^{55,56} Specifically, the analog with 3,4-dichlorophenyl substituent in this position (**5**) had improved activity against *Pf*. In our own work, we previously explored Fos/FR900098 analogs that bind to both the DXP binding site and the adjacent NADPH binding site through extension of the retrohydroxamate with either an *N*-acyl or *N*-alkoxy substituent (**4**).^{34,38,54} Here, we report a series of FR900098 analogs that combine features of both of these efforts: an α -3,4-dichlorophenyl group and an *N*-alkoxy substituent (**6** and **7**). Aromatic *N*-alkoxy substituents were chosen in order to explore binding to the NADPH binding site, as in earlier work.^{34,54} The phosphonic acid salts (**6**) and

pivaloyloxymethylene prodrugs (**7**) were made to explore both enzyme inhibition and cellular activity.

Results & discussion

Synthesis

The route used to prepare the compounds is shown in Scheme 1. Starting from 3,4-dichlorobenzaldehyde, compound **8** was synthesized as previously described.⁵⁵ Compound **8** was then subjected to reductive amination to yield benzyloxyamine **9**. Upon acetylation, the retrohydroxamate moiety was completed to give the diethyl ester phosphonate **10**. The diethyl ester of **10** was cleaved using trimethylsilylbromide (TMSBr), and the dipivaloyloxymethyl (diPOM) prodrug **11** was synthesized using pivaloyloxymethylchloride (POMCl). Compound **11** was debenzylated with boron trichloride (BCl₃) to give compound **12**. The hydroxylamine of compound **12** was then reacted with the appropriate alkyl bromide to undergo Williamson ether synthesis, yielding diPOM prodrug **7l**. For the remaining ethers, the benzyl protecting group of the hydroxylamine in compound **10** was cleaved using BCl₃ to give intermediate **13**. Application of Williamson ether conditions with commercially available alkyl bromides yielded *N*-*O*-ethers



Scheme 1 Synthesis of FR900098 Analogs. Reagents and conditions: (a) (i) OBHA, MeOH, rt, (ii) NaBH₃CN, acetic acid, MeOH, rt, 41%; (b) AcCl, Et₃N, DCM 0 °C to rt, 85%; (c) BCl₃, DCM, -78 °C, 95–97%; (d) alkyl bromide, NaH, THF, 0 °C to rt, 4–97%; (e) (i) TMSBr, DCM, 0 °C to rt, (ii) 7M NH₃ in MeOH, 57%-quantitative; (f) 4-isopropylphenyl boronic acid, Pd(PPh₃)₄, diethyl ether, Na₂CO₃, EtOH, 70 °C, 19%; (g) POMCl, DIPEA, NaI, THF, 60 °C, 13–60%; (h) (i) TMSBr, DCM, 0 °C to rt, (ii) POMCl, DIPEA, NaI, THF, 60 °C, 15%. Abbreviations: OBHA = *O*-benzyloxyamine, AcCl = acetyl chloride, RBr = alkyl bromide, TMSBr = trimethylsilylbromide, POMCl = pivaloyloxymethylchloride, DCM = dichloromethane, DIPEA = *N,N*-diisopropylethylamine, THF = tetrahydrofuran.



14b–h,j–n. Compound **14h** was subjected to Suzuki coupling to give compound **14i**. Diethyl ester phosphonate **13** and *N*-alkoxy compounds **14b–n** were converted to diammonium salts **6a–n** via cleavage of the diethyl groups with TMSBr followed by treatment with 7 M NH₃ in methanol. Reaction of salts **6a–k,m,n** with POMCl yielded diPOM ester phosphonates **7a–k,m,n**.

Biological evaluation

The compounds were subjected to a series of assays to evaluate their activity as inhibitors of DXR, their ability to inhibit *Mtb* and *Pf* growth, and their cytotoxicity in mammalian cells. In addition, to verify that the compounds inhibit the MEP pathway as expected, malaria parasites treated with the most active compounds were subjected to IPP rescue, as supplementation with IPP restores growth to parasites treated with well-characterized MEP pathway inhibitors.⁵⁷ These compounds were also examined against *Pf* parasites bearing the *had1* mutation, which is resistant to fosmidomycin due to increased substrate levels.⁵⁸

Evaluation of phosphonic acid salts against *Pf*DXR and *Mtb*DXR

Salts **6a–n** were tested for activity against both *Pf*DXR and *Mtb*DXR, and this data is shown in Table 1. Enzyme activity

was initially measured at a single concentration of 100 μM. For compounds inhibiting DXR more than 90%, the half-maximal inhibitory concentration (IC₅₀) was determined (Fig. S1 and S2†). Previous docking studies showed that aromatic *N*-alkoxy substituents could occupy the NADPH cofactor binding site, and a series of aromatic *N*-*O*-ethers were most successful.^{13,14} To explore the effect of adding a 3,4-dichlorophenyl substituent (alpha to the phosphorous atom) to the previously synthesized *N*-*O*-ethers, compounds **6b–6i** were synthesized.

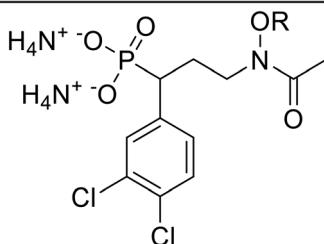
Among the first set of nine analogs (**6a–i**), compounds **6a** and **6e** were the most potent against *Pf*DXR with IC₅₀ values of 13.62 and 16.83 μM, respectively. Interestingly, compound **6e** is the only analog of the initial set which bears a methyl branch at the benzyl position of the *N*-*O*-ether. SAR exploration of the phenyl ring of **6e** yielded compounds **6j–n**. Of these five 4-position analogs, three showed improved *Pf*DXR activity relative to **6e**. Compound **6k** (4-OCH₃) displayed an IC₅₀ value against *Pf*DXR of 1.21 μM. Although not as active as parent compounds fosmidomycin (**1**) and FR900098 (**2**), compound **6l** (4-OCH₃) and **6n** (4-F) were the quite potent against *Pf*DXR with IC₅₀ values of 0.11 μM and 0.47 μM, respectively.

When evaluated for activity against *Mtb*DXR, the majority of salts **6a–n** were inactive. Two exceptions were compounds **6l** (4-OCH₃) and **6n** (4-F), with IC₅₀ values of 0.78 μM and 4.78 μM, respectively. Interestingly, the IC₅₀

Table 1 *In vitro* activity of phosphonic acid salts **6a–6n**

Compound	R	<i>Pf</i> DXR ^a	<i>Pf</i> IC ₅₀ ^a	<i>Mtb</i> DXR ^a	<i>Mtb</i> MIC ^b	clog P ^c
		μM	μM	μM	μg mL ⁻¹ (μM)	
1 (Fos)	H	0.063 (ref. 59)	1.021 (ref. 59)	0.44 (ref. 34)	>500	−2.64
2 (FR)	H	0.022 (ref. 59)	0.511 (ref. 59)	2.91 (ref. 34)	>500	−2.54
6a	H	13.62	18.63 ± 3.81	(15.70)	>100 (>266)	0.31
6b	CH ₂ Ph	(26.66)	31.46 ± 7.85	(34.20)	>100 (>214)	3.06
6c	CH ₂ -(2-Naphthyl)	(46.64)	60.91 ± 4.97	(82.10)	12.5 (24.2)	4.06
6d	CH ₂ -4-(1,1'-Biphenyl)	(26.97)	86.37 ± 10.12	(93.90)	12.5 (23.1)	4.72
6e	CH(CH ₃)Ph	16.83	3.60 ± 0.80	(64.60)	>100 (>208)	3.47
6f	CH ₂ -(4- <i>i</i> Pr)Ph	26.46	68.73 ± 9.44	(80.90)	6.25 (12.3)	4.29
6g	(CH ₂) ₄ Ph	(25.64)	63.31 ± 16.73	(99.10)	1.56 (3.1)	3.86
6h	CH ₂ -(4-Br)Ph	(53.07)	67.82 ± 3.57	(72.30)	>50 (>92)	3.83
6i	CH ₂ -4-[4'- <i>i</i> Pr-(1,1'-biphenyl)]	(56.11)	38.14 ± 0.43	(63.20)	25 (45)	4.72
6j	CH(CH ₃)-(4-Cl)Ph	20.35	68.59 ± 20.90	(66.20)	37 (72)	3.62
6k	CH(CH ₃)-(4-CH ₃)Ph	1.21	3.53 ± 0.66	(30.70)	>50 (>101)	3.56
6l	CH(CH ₃)-(4-OCH ₃)Ph	0.11	0.30 ± 0.04	0.78	>50 (>98)	2.86
6m	CH(CH ₃)-(4-CF ₃)Ph	(74.69)	79.24 ± 12.11	(59.60)	>50 (>91)	3.94
6n	CH(CH ₃)-(4-F)Ph	0.47	2.42 ± 0.43	4.78	>100 (>200)	3.62

^a Half-maximal inhibitory activity (IC₅₀). Values in parentheses are percent remaining enzyme activity at 100 μM. ^b 7H9/glucose/casitone/tyloxapol media. ^c Calculated using MarvinSketch.



value of **6l** is a significant improvement over FR900098 (**2**) and on par with that of fosmidomycin (**1**). The increase in potency for these new compounds could be due to the increased electron density of the *para* substituents. Additional analogs will be needed to show a clear trend.

Evaluation of phosphonic acid salts against *P. falciparum* and *M. tuberculosis*

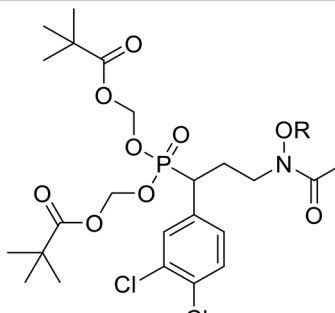
Compounds **6a–n** were also evaluated for activity against *Pf*3D7 malaria parasites and *Mtb*H37Rv (Table 1, Fig. S3†). Among the first set of compounds (**6a–i**), compound **6e** was again the most potent against *Pf* (IC₅₀ value of 3.60 μM). This data is further evidence that the branch methyl group is beneficial for activity. Without the branch methyl group, potency suffers as seen in compound **6b**. Among compounds **6j–6n**, compounds **6k**, **6l**, and **6n** had improved activity compared with the parent compound **6e**. This trend is similar to the compounds' activities against the enzyme. Compound **6l** is the most potent phosphonic acid salt of the series against *Pf* parasites, with an IC₅₀ value of 0.30 μM, and the only analog with potency against *Pf* parasites better than that of fosmidomycin (**1**). Against *Mtb*, several compounds showed modest activity,

which is an improvement over both parent compounds. The most potent salt was compound **6g**. None of these salts, however, were active against *Mtb*DXR, indicating the compounds likely inhibit *Mtb* growth through an alternate mechanism.

Evaluation of analog prodrugs against *P. falciparum* and *M. tuberculosis*

Prodrugs **7a–n** were evaluated for activity against *Pf*3D7 parasites and *Mtb* cells (Table 2 and Fig. S3†). Compound **7l**, the prodrug of phosphonic acid **6l**, is the most potent prodrug against *Pf* parasites with an IC₅₀ value of 0.37 μM. This compound is more active against *Pf* parasites than either fosmidomycin (**1**) or FR900098 (**2**). Further, this data confirms the branch methyl group and the 4-OCH₃ substituent are beneficial for the potency of these compounds. When evaluated for activity against *Mtb*, several prodrugs had moderate activity. Prodrugs **7a**, **7l**, and **7n** had the greatest potency with MIC values of 4.60 μg mL^{−1} (6.5–8.1 μM). This is a significant improvement in activity compared with the parent salts which were inactive, confirming the lipophilic prodrug is important for activity, likely due to increased cell penetration.

Table 2 *In vitro* activity of POM-prodrug compounds **7a–7n**



Compound	R	<i>Pf</i> IC ₅₀ ^a	<i>Mtb</i> MIC ^b	clog <i>Pf</i> ^c
		μM	μg mL ^{−1} (μM)	
1 (Fos)	H	1.021 (ref. 59)	>500 (ref. 34)	−2.64
2 (FR)	H	0.511 (ref. 59)	>500 (ref. 34)	−2.54
7a	H	13.01 ± 0.03	4.60 (8.1)	5.59
7b	CH ₂ Ph	41.60 ± 4.20	6.25 (9.5)	7.65
7c	CH ₂ -(2-naphthyl)	13.96 ± 1.91	6.25 (8.8)	8.65
7d	CH ₂ -4-(1,1'-biphenyl)	8.78 ± 0.92	12.50 (17.0)	9.77
7e	CH(CH ₃)Ph	5.22 ± 1.33	9.40 (13.9)	8.06
7f	CH ₂ -(4- <i>i</i> Pr-Ph)	13.86 ± 1.79	12.50 (17.8)	8.88
7g	(CH ₂) ₄ Ph	13.94 ± 0.92	12.50 (17.8)	8.77
7h	CH ₂ -(4-Br)Ph	34.80 ± 6.80	6.25 (8.5)	8.42
7i	CH ₂ -4-[4'- <i>i</i> Pr-(1,1'-biphenyl)]	14.92 ± 5.55	>100 (>128)	10.54
7j	CH(CH ₃)-(4-Cl)Ph	30.07 ± 6.64	9.40 (13.3)	9.02
7k	CH(CH ₃)-(4-CH ₃)Ph	31.91 ± 8.09	12.50 (18.2)	8.97
7l	CH(CH ₃)-(4-OCH ₃)Ph	0.37 ± 0.06	4.60 (6.5)	8.25
7m	CH(CH ₃)-(4-CF ₃)Ph	23.84 ± 3.91	>100 (>134)	9.39
7n	CH(CH ₃)-(4-F)Ph	21.16 ± 1.15	4.60 (6.6)	8.21

^a *Pf*3D7 parasites. ^b *Mtb* H37Rv; 7H9/glucose/casitone/tyloxapol media. ^c Calculated using MarvinSketch; *Pf* = *P. falciparum*; *Mtb* = *M. tuberculosis*; IC₅₀ = half-maximal inhibitory concentration; MIC = minimal inhibitory concentration.



Confirming on-target MEP pathway inhibition: IPP rescue and *had1* mutant resistance

The most active inhibitors of *Pf*DXR and *Pf* parasite growth were further examined to confirm inhibition of the MEP pathway. The IPP rescue assay is a useful method of determining whether inhibitors are acting through the MEP pathway. Inhibition of DXR stops production of IPP, blocking the entire MEP pathway, and killing the parasite. In the presence of media supplemented with excess IPP, parasites are rescued from inhibition and growth is restored. The most potent antiparasitic compounds were evaluated in the IPP rescue assay (Fig. 3). The addition of exogenous IPP rescues the growth of *P. falciparum* parasites treated with phosphonic acid salts **6k**, **6l**, **6n**, and prodrug **7l**. This data suggests these analogs inhibit *Pf* parasite growth *via* blocking the MEP pathway.

IPP rescue alone does not fully confirm MEP pathway inhibition as compounds with alternate mechanisms of action, related to the apicoplast but not the MEP pathway, can also be rescued by IPP.⁵⁷ To fully confirm the mechanism of action of **6k**, **6l**, **6n**, and **7l** is through inhibition of the MEP pathway, they were tested for cross resistance to the

fosmidomycin-resistant strain of *Pf* bearing the *had1* mutation. This mutation confers fosmidomycin resistance *via* increased levels of DXR substrate DXP. Indeed, compounds **6k**, **6l**, **6n**, and **7l** also displayed increased resistance to *had1* mutant *Pf* strain (Fig. S3 and S4†).

Cytotoxicity

Compounds that had the most potent activity against *Pf* parasites (**6k**, **6l**, **6n**, and **7l**) were evaluated for cytotoxic properties against human hepatocytes (HepG2, Table 3). None of the compounds were cytotoxic up to 100 $\mu\text{g mL}^{-1}$.

Conclusion

We set out to design a novel set of FR900098 analogs based on compounds **4** and **5**, which have reported potent activities against *P. falciparum* and *M. tuberculosis*. This work complements our prior work by asking if combining the α -3,4-dichlorophenyl and *N*-alkoxyaryl substituents would enhance activity against *Pf*DXR, *Mtb*DXR, and the intact organisms. We also questioned if a prodrug approach would impact the potency of the compounds. Finally, we queried

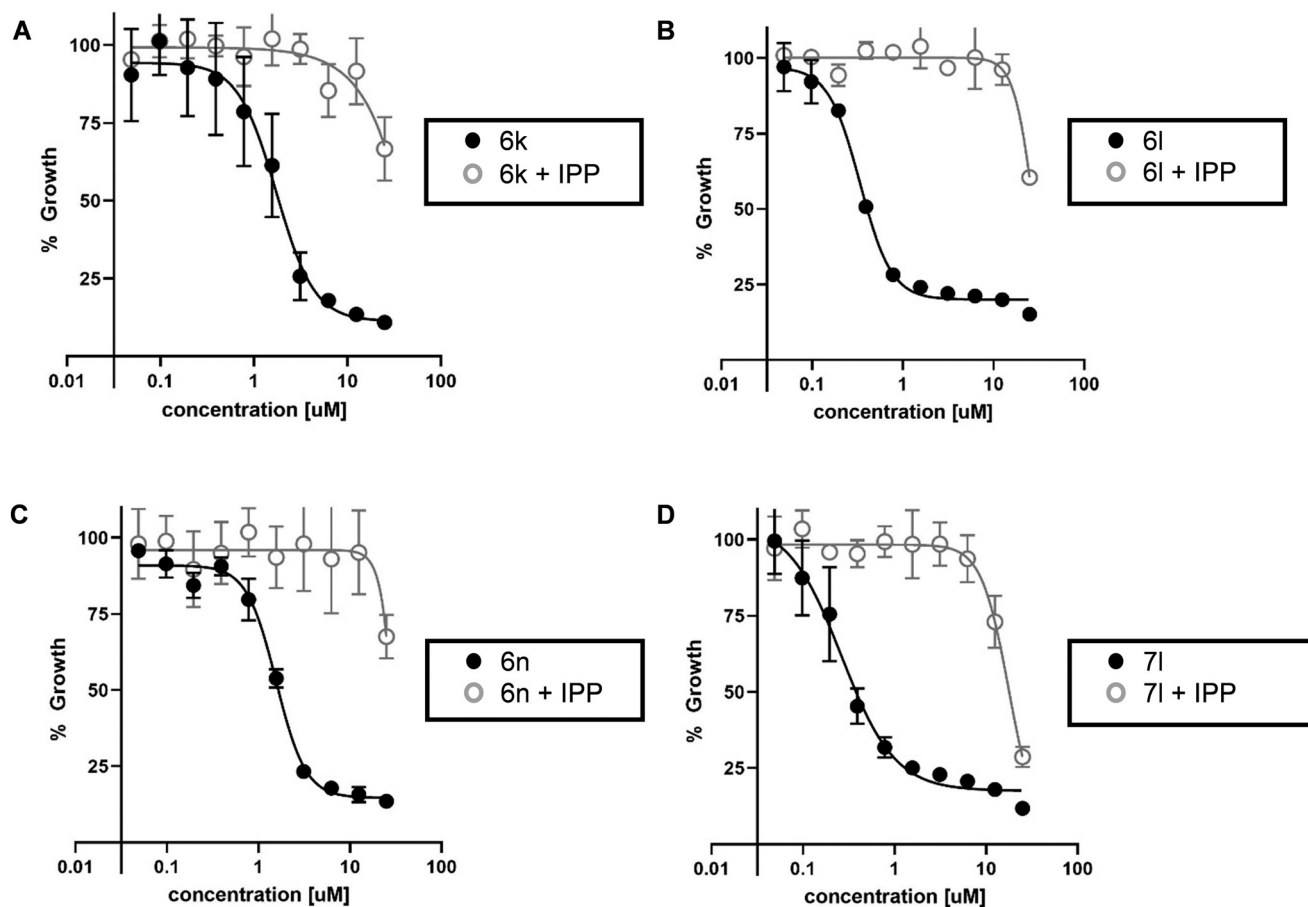
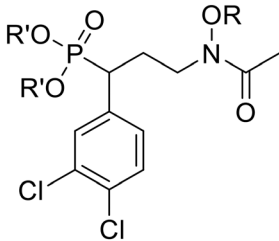


Fig. 3 IPP rescue of inhibitor-treated *P. falciparum*. Drug treatment kills *Pf* parasites (closed circles). IPP, the product of the MEP pathway, rescues growth of drug-treated parasites (open circles). Shown are graphs from four independent experiments: A) **6k**; B) **6l**; C) **6n**; D) **7l**.



Table 3 Cytotoxicity of analogs **6k**, **6l**, **6n**, and **7l**


Compound	R	R'	HepG2 IC ₅₀ μg mL ⁻¹ (μM)
6k	CH(CH ₃)-(4-CH ₃)Ph	NH ₄	>100 (>200)
6l	CH(CH ₃)-(4-OCH ₃)Ph	NH ₄	>100 (>200)
6n	CH(CH ₃)-(4-F)Ph	NH ₄	>100 (>200)
7l	CH(CH ₃)-(4-OCH ₃)Ph	POM	>100 (>140)
Tamoxifen	—	—	11.72 μM

IC₅₀ = half-maximal inhibitory concentration.

whether our most potent compounds were on-target inside the cell, while also evaluating their cytotoxicity.

The data shows that the disubstituted α -3,4-dichlorophenyl, *N*-alkoxyaryl analogs are most potent against *Pf*DXR and *Pf* parasites. The 4-OCH₃ (**6l**) and 4-F (**6n**) compounds were the most potent salts with IC₅₀ values against *Pf*DXR of 0.11 and 0.47 μM, respectively, and with IC₅₀ values against *Pf* parasites of 0.30 and 2.42 μM, respectively. We speculate that slightly lower log *P* values of these compounds, compared to the rest of the series, play a role in their enhanced activities. Interestingly, prodrugs of these compounds did not show a robust improvement in activity against *Pf* parasites. Aside from **7l**, all of the prodrug compounds displayed a dramatic reduction in antimalarial potency relative to their salt parent compounds. This may be due to insufficient deprotection of the bisphosphonate ester by *Plasmodium* spp. Indeed, steric hindrance due to the α -3,4-dichlorophenyl substituent could prevent or slow esterase activity on these compounds. Nonetheless, compound **7l** (4-OCH₃, IC₅₀ *Pf*3D7 = 0.37 μM), was very potent against *Pf* parasites, exceeding the activity of parent compounds fosmidomycin (**1**) and FR900098 (**2**).

Interestingly, most of the phosphonic acid salts (**6a–6n**) were only weakly active against *Mtb*DXR. The most active compounds were again **6l** and **6n** at 0.78 and 4.78 μM, respectively. One reason for this lower activity could be explained by an earlier observation. Andaloussi *et al.* stated that addition of the α -3,4-dichlorophenyl group could clash with a Trp residue near the binding site of *Mtb*DXR, resulting in lower inhibition of the protein.³³ Many of the salt compounds displayed weak activity against *Mtb* cells. Because most of these compounds did not inhibit *Mtb*DXR, it is presumed that their cellular activity is through an alternate target. Prodrugging the phosphonates led to improved *Mtb* cellular activity. For compounds **7l** and **7n** (MIC value of 4.60 μg mL⁻¹ for both analogs), this activity may be due to DXR inhibition. The activity of these compounds, as well as salt **6l**, exceeds the activity of fosmidomycin (**1**) and FR900098 (**2**).

Compounds displaying the most potent activity against *Pf*DXR and *Pf* parasites were examined further to determine if their mechanism of action was through the MEP pathway. Drug-treated parasites were effectively rescued by addition of exogenous IPP, the product of the MEP pathway. Similar to known MEP pathway inhibitor fosmidomycin, IPP rescued parasites from treatment with compounds **6k**, **6l**, **6n**, and **7l**, confirming the intracellular target.^{19,37} Additionally, these compounds displayed cross resistance to the *had1* mutant strain of *Pf*, mimicking fosmidomycin again. Thus, these data confirm the on-target mechanism of action of these compounds as MEP pathway inhibitors. Finally, these four most active compounds were tested for cytotoxicity against human HepG2 cells and found to display negligible cytotoxicity.

Thus, taken together, the data show that FR900098 analogs bearing both the α -3,4-dichlorophenyl and *N*-branched-alkoxyaryl substituents are very potent compounds against *Pf*DXR and *Pf* parasites. Compounds **6k**, **6l**, **6n**, and **7l** are especially effective, and the prodrug is not required for high antimalarial activity. The compounds have an on-target mechanism of action and display low human cytotoxicity. While it is clear that the activity of these compounds against *Mtb*DXR and *Mtb* cells is not straightforward, future studies will focus on refinement of these structures and further biological evaluation as antimalarial compounds.

Experimental methods

General

All reagents were purchased from commercial suppliers and used without further purification. Anhydrous solvents were purified by MBRAUN MB-SPS solvent purification system before use. Air sensitive reactions were carried out under a nitrogen atmosphere. ¹H and ¹³C NMR spectra were recorded in CDCl₃ or C₂D₆OS on an Agilent spectrometer at 400 MHz or 101 MHz, respectively, with TMS, H₂O, or solvent signal as



internal standard. Chemical shifts are given in parts per million (ppm). Spin multiplicities are given with the following abbreviations: s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), t (triplet), dt (doublet of triplets), ddt (doublet of doublet of triplets), q (quartet), qt (quintet), and m (multiplet). Mass spectra were measured in the ESI mode on an HPLC-MS (Shimadzu LCMS-2020) or in the EI mode on an GC-MS (Shimadzu GCMS-QP2010S). Thin layer chromatography (TLC) was performed on Baker-flex Silica Gel IB2-F silica plates. The purity of synthesized compounds (>95%) was determined by HPLC-MS (Shimadzu LCMS-2020) using Shimadzu Nexcol C18 5 μ m particle (50 $\times$ 3.0 mm). A linear gradient of acetonitrile (50–100%) in water (containing 0.1% formic acid) was used, at a flow rate of 0.2 mL min⁻¹ from 0.5 to 15.5 min, followed by 100% acetonitrile for an additional 0.5 min. Quantification of compound purity was determined *via* integration of the area under the DAD curve using the LabSolutions software. High-resolution mass spectroscopy spectra (HRMS) were recorded in positive or negative ESI mode on a Waters Q-TOF Ultima mass spectrometer (UIUC Mass Spectrometry Laboratory) or in positive FAB mode on a VG Analytical VG70SE magnetic sector mass spectrometer (JHU Mass Spectrometry Facility).

(2E)-3-(3,4-Dichlorophenyl) prop-2-enal. A solution of 3,4-dichlorophenyl benzaldehyde (5.45 g, 31.1 mmol) and triphenylphosphoranylidene acetaldehyde (10.42 g, 34.3 mmol) in 200 mL anhydrous toluene under nitrogen was stirred at 50 °C for 1 hour and at 80 °C for 72 hours. The toluene was removed under reduced pressure. The crude residue was purified *via* column chromatography (1:1 dichloromethane:hexanes) to afford a slightly yellow solid (4.04 g, 65%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 6.68 (dd, 1H), 7.37 (d, 1H), 7.45 (m, 3H), 9.71 (d, 1H).

Diethyl [1-(3,4-dichlorophenyl)-3,3-diphenoxypropyl] phosphonate. To a flask containing (2E)-3-(3,4-dichlorophenyl) prop-2-enal (4.04 g, 20.1 mmol) were added phenol (4.92 g, 52.2 mmol) and triethylphosphite (4.18 g, 25.1 mmol) under nitrogen. The mixture was heated on an oil bath at 100 °C for 48–72 hours. The excess triethylphosphite was removed under reduced pressure. The crude residue was purified *via* column chromatography (3:2 hexanes:ethyl acetate) to yield a yellow oil (6.91 g, 67%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.13 (t, 3H), 1.25 (t, 3H), 2.51 (m, 1H), 2.76 (m, 1H), 3.32–3.45 (m, 1H), 3.74–3.87 (m, 1H), 3.89–4.17 (m, 2H), 5.68 (dd, 1H), 6.82–6.93 (m, 3H), 6.95–7.03 (m, 2H), 7.17–7.26 (m, 6H), 7.36–7.47 (m, 2H).

Diethyl [1-(3,4-dichlorophenyl)-3-oxopropyl] phosphonate (8). To a solution of diethyl [1-(3,4-dichlorophenyl)-3,3-diphenoxypropyl] phosphonate (3.88 g, 7.62 mmol) in 73 mL acetone were added 8.2 mL of 2 M HCl acid and 5.4 mL of water. The mixture heated on an oil bath at 70 °C for 48 hours. After removing the acetone under reduced pressure, the residue was dissolved in dichloromethane (50 mL) and washed with distilled water (2 $\times$ 50 mL). The organic phase was dried over sodium sulfate and filtered. Dichloromethane

was removed under reduced pressure. The crude residue was purified *via* column chromatography (3:2 dichloromethane:ethyl acetate) to afford a colorless oil as the product (2.02 g, 78%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.11 (t, 3H), 1.23 (t, 3H), 2.99–3.26 (m, 2H), 3.59–3.74 (ddd, 1H), 3.79–3.89 (m, 1H), 3.91–3.99 (m, 2H), 4.00–4.13 (m, 2H), 7.12–7.52 (m, 3H), 9.66 (s, 1H).

O-Benzylhydroxylamine. Sodium hydroxide (0.24 g, 5.95 mmol) and O-benzylhydroxylamine hydrochloride (0.95 g, 5.95 mmol) were dissolved in 30 mL of diethyl ether and 10 mL of distilled water. The mixture was stirred for 0.5 hours at room temperature. The aqueous phase was then extracted with diethyl ether (3 $\times$ 50 mL). The organic phase was dried over sodium sulfate and filtered. Diethyl ether was removed under reduced pressure to yield a colorless liquid (0.72 g, quantitative). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 4.67 (s, 2H), 5.09 (bs, 2H), 7.35 (m, 6H).

Diethyl {3-[(benzyloxy) amino]-1-(3,4-dichlorophenyl) propyl} phosphonate (9). To a solution of 8 (2.34 g, 6.90 mmol) in 70 mL anhydrous methanol was added O-benzylhydroxylamine (0.93 g, 7.59 mmol) under nitrogen and stirred at room temperature overnight. To the reaction mixture were added sodium cyanoborohydride (1.52 g, 24.1 mmol) and 1.97 mL of acetic acid. The mixture stirred at room temperature for three hours. The methanol was removed under reduced pressure. The crude residue was dissolved in dichloromethane and washed with saturated NaHCO₃ (60 mL) until the aqueous phase was slightly basic. The aqueous phase was then extracted with dichloromethane (5 $\times$ 50 mL). The combined organic phases were dried over sodium sulfate and filtered. The dichloromethane was removed under reduced pressure. The crude product was purified *via* column chromatography (97:3 dichloromethane:methanol) and yielded a colorless oil (1.28 g, 42%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.15 (t, 3H), 1.28 (t, 3H, *J* = 7.6 Hz), 1.95–2.13 (m, 1H), 2.27–2.42 (m, 1H), 2.58–2.74 (m, 1H), 2.79–2.90 (m, 1H), 3.11–3.27 (ddd, 1H), 3.76–3.88 (m, 1H), 3.90–3.98 (m, 1H), 4.01–4.12 (m, 2H), 4.54–4.76 (s, 2H), 5.49 (bs, 1H), 7.01–7.20 (m, 1H), 7.23–7.45 (m, 7H). LCMS (ESI⁺): 446.25 *m/z* [M + H]⁺.

Diethyl {3-[N-(benzyloxy) acetamido]-1-(3,4-dichlorophenyl) propyl} phosphonate (10, 14b). A solution of 9 (1.51 g, 3.93 mmol) in anhydrous dichloromethane (34 mL) under nitrogen was cooled to 0 °C. Triethylamine (0.686 g, 6.78 mmol) and acetyl chloride (0.319 g, 4.07 mmol) were added to the reaction mixture at 0 °C. The mixture was warmed to room temperature and allowed to stir overnight. The reaction was quenched with water. The aqueous phase was extracted with dichloromethane (3 $\times$ 70 mL). The organic phase was dried over sodium sulfate and filtered. After dichloromethane was removed under reduced pressure, the crude product was purified *via* column chromatography (100% ethyl acetate). The desired product was obtained as a slightly yellow oil (1.40 g, 85%). Spectroscopic values match those previously reported.⁵⁶ ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.15 (t, 3H), 1.27 (t, 3H), 2.03 (s, 3H), 2.09–2.3 (m, 1H), 2.34–2.50 (m, 1H),



2.93–3.08 (ddd, 1H), 3.39–3.60 (m, 2H), 3.76–3.88 (m, 1H), 3.90–3.98 (m, 1H), 3.99–4.11 (m, 2H), 4.72 (s, 2H), 7.12–7.44 (m, 8H). LCMS (ESI⁺): 488.25 *m/z* [M + H]⁺.

[[{3-[*N*-(Benzyloxy)acetamido]-1-(3,4-dichlorophenyl)propyl}({[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl]oxy]methyl 2,2-dimethylpropanoate (11, 7b). A solution of **10** (0.047, 0.088 mmol) in anhydrous dichloromethane (1 mL) under nitrogen was cooled to 0 °C. Trimethylsilyl bromide (0.041 g, 0.265 mmol) was added dropwise to the solution under nitrogen. The mixture was warmed to room temperature and stirred overnight. Trimethylsilyl bromide and dichloromethane were removed under reduced pressure. The crude residue was stirred at room temperature in anhydrous methanol for one to two hours. The methanol was removed under reduced pressure. To a solution of the crude residue in THF were added *N,N*-diisopropylethylamine (0.042 g, 0.324 mmol) and pivaloyloxymethyl chloride (0.731 g, 4.86 mmol) at room temperature. The reaction mixture was heated to 60 °C and stirred for 48–72 hours. The dimethylformamide was removed under reduced pressure. The crude residue was dissolved in dichloromethane and washed with saturated NaHCO₃ (10 mL), followed by brine (15 mL). The organic phase was dried over anhydrous sodium sulfate and filtered. The dichloromethane was removed under reduced pressure and the residue was purified *via* column chromatography, to afford slightly yellow oil (0.850 g, 60%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.18–1.20 (d, 18H), 2.00 (s, 3H), 2.17 (bs, 1H), 2.27–2.48 (m, 1H), 3.05–3.20 (m, 1H), 3.37–3.55 (m, 2H), 4.71 (s, 2H), 5.43–5.68 (dd, 4H), 7.12 (s, 1H), 7.28 (s, 2H), 7.37 (s, 5H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.1, 26.0, 26.5, 38.4, 41.0, 43.1, 76.4, 81.5, 128.5, 128.6, 128.8, 128.9, 129.0, 130.3, 130.9, 131.7, 132.4, 133.9, 134.2, 134.3, 172.0, 176.2. HRMS (ESI⁺) calculated for C₃₀H₄₃Cl₂NO₉P 660.1896, found 660.1901 [M + H]⁺. Purity = 98.1%.

[[{1-(3,4-Dichlorophenyl)-3-(*N*-hydroxyacetamido)propyl}({[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl]oxy]methyl 2,2-dimethylpropanoate (12, 7a). A solution of **11** (1 eq.) anhydrous dichloromethane (0.1 M) under nitrogen was cooled to –80 °C. To the solution was added boron trichloride (1 M in dichloromethane) (3 eq.) dropwise. The reaction mixture was stirred at –80 °C for four to five hours and then was quenched with cold saturated NaHCO₃. The aqueous phase was extracted with ethyl acetate (3 × 15 mL). The organic phases were combined and dried over sodium sulfate and filtered. The ethyl acetate was removed under reduced pressure. The crude product was purified *via* column chromatography (97:3 dichloromethane:methanol), to afford a slightly yellow oil (0.200 g, 46%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.21 (s, 18H), 1.90–2.02 (m, 3H), 2.08–2.40 (m, 2H), 2.40–2.63 (m, 1H), 3.00–3.24 (m, 1H), 3.26–3.56 (m, 2H), 3.67–4.08 (m, 1H), 5.25–5.68 (m, 4H), 7.18 (bs, 1H), 7.37–7.41 (m, 3H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.1, 25.9, 26.5, 38.4, 41.0, 43.0, 81.4, 128.5, 128.9, 130.6, 131.6, 132.5, 134.1, 171.9, 176.1. HRMS (ESI⁺) calculated for C₂₃H₃₅Cl₂NO₉P 570.1426, found 570.1420 [M + H]⁺. Purity = 97.3%.

Diethyl [1-(3,4-dichlorophenyl)-3-(*N*-hydroxyacetamido)propyl] phosphonate (13). A solution of **10** (1.37 g, 2.81 mmol) in anhydrous dichloromethane (28 mL) under nitrogen was cooled to –80 °C. To the solution was added boron trichloride (1 M in dichloromethane) (14.5 mL, 14.4 mmol) dropwise. The reaction mixture was stirred at –80 °C for four to five hours and then was quenched with cold saturated NaHCO₃. The aqueous phase was extracted with ethyl acetate (4 × 60 mL). The organic phases were combined and dried over sodium sulfate and filtered. The ethyl acetate was removed under reduced pressure. The crude product was purified *via* column chromatography (97:3 dichloromethane:methanol), to afford an orange, viscous oil (1.15 g, 97%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.15–1.36 (m, 6H), 1.87 (m, 1H), 2.08 (s, 3H), 2.43 (m, 1H), 3.12 (m, 1H), 2.79–2.90 (m, 1H), 3.42 (m, 1H), 3.68 (m, 1H), 3.90–4.11 (m, 4H), 7.13–7.24 (m, 1H), 7.38–7.49 (m, 2H), 9.45 (bs, 1H). LCMS (ESI⁺): 398.25 *m/z* [M + H]⁺.

General procedure for synthesis of *N*-O-ethers 14b–h,j–n

Compound **13** (1 eq.) was dissolved in anhydrous tetrahydrofuran (0.1 M) under a nitrogen atmosphere and cooled to 0 °C. Sodium hydride (1.2 eq.) was added to the solution, and the reaction was stirred for 20 minutes. To the reaction mixture was added aryl chloride (1.2 eq.). The mixture was warmed to room temperature and stirred for 18–48 hours. After removing the tetrahydrofuran under reduced pressure, the residue was dissolved in dichloromethane and quenched with water. The aqueous phase was extracted with dichloromethane (5 × 20 mL). The organic phase was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure. The crude product was purified *via* column chromatography (97:3 dichloromethane:methanol) or silica gel preparatory plate (97:3 dichloromethane:methanol) to give the pure compound.

Diethyl [1-(3,4-dichlorophenyl)-3-{*N*-[(naphthalen-2-yl)methoxy]acetamido}propyl] phosphonate (14c). Slightly yellow oil (0.209 g, 75%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.13 (t, 3H), 1.25 (t, 3H), 2.07 (s, 3H), 2.18 (m, 1H), 2.46 (m, 1H), 2.94–3.08 (m, 1H), 3.41–3.62 (m, 2H), 3.76–3.86 (m, 1H), 3.88–3.96 (m, 1H), 3.97–4.08 (m, 2H), 4.80–4.95 (s, 2H), 7.07–7.16 (m, 1H), 7.23–7.43 (m, 3H), 7.46–7.57 (m, 2H), 7.71–7.89 (m, 4H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.3, 20.5, 26.8, 40.8, 42.1, 62.2, 62.3, 62.7, 62.8, 126.3, 126.6, 126.7, 126.8, 128.0, 128.6, 128.6, 128.6, 128.7, 130.4, 130.4, 131.0, 131.1, 133.1, 133.4, 135.8, 135.9. LCMS (ESI⁺): 538.25 *m/z* [M + H]⁺. Purity = 99.1%.

Diethyl {3-[*N*-({[1,1'-biphenyl]-4-yl)methoxy}acetamido]-1-(3,4-dichlorophenyl)propyl} phosphonate (14d). Slightly yellow oil (0.086 g, 63%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.15 (t, 3H), 1.27 (t, 3H), 1.69 (m, 1H), 2.07 (s, 3H), 2.183 (m, 1H), 2.45 (m, 1H), 2.98 (ddd, 1H), 3.43–3.62 (m, 2H), 3.77–3.86 (m, 1H), 3.89–3.97 (m, 1H), 3.98–4.14 (m, 2H), 4.75 (s, 2H), 7.13–7.29 (m, 2H), 7.30–7.51 (m, 6H), 7.55–7.66 (m, 4H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.3, 20.4, 26.8, 62.7,



62.8, 127.1, 127.4, 127.7, 128.7, 128.9, 129.6, 130.4, 131.0, 165.6. LCMS (ESI⁺): 564.25 *m/z* [M + H]⁺. Purity = 99.6%.

Diethyl [1-(3,4-dichlorophenyl)-3-[N-(1-phenylethoxy)acetamido]propyl] phosphonate (14e). Slightly yellow oil (0.480 g, 38%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.11–1.16 (q, 3H), 1.24–1.29 (t, 3H), 1.52 (q, 4H), 1.97 (m, 4H), 2.29 (m, 1H), 2.85 (m, 2H), 3.49 (m, 1H), 3.93 (m, 3H), 4.69 (s, 1H), 7.09–7.34 (m, 8H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.3, 20.3, 26.2, 40.7, 42.1, 62.2, 62.7, 82.9, 127.1, 127.2, 128.6, 128.6, 128.8, 130.3, 130.9, 131.1. LCMS (ESI⁺): 502.25 *m/z* [M + H]⁺. Purity = 99.1%.

Diethyl [1-(3,4-dichlorophenyl)-3-[N-[[4-(propan-2-yl) phenyl] methoxy] acetamido] propyl] phosphonate (14f). Slightly yellow oil (0.070 g, 40%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.16 (t, 6H), 1.26 (d, 6H), 1.633 (s, 1H), 2.04 (s, 3H), 2.17 (m, 1H), 2.44 (m, 1H), 2.86–3.09 (m, 1H), 3.40–3.62 (m, 2H), 3.76–3.87 (m, 1H), 3.90–3.98 (m, 1H), 3.98–4.13 (m, 2H), 4.61–4.75 (s, 2H), 6.73–7.09 (m, 3H), 7.09–7.30 (m, 3H), 7.32–7.46 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.4, 20.4, 23.9, 26.7, 33.9, 40.7, 42.1, 62.2, 62.756, 126.8, 128.7, 129.3, 130.4, 131.1, 131.4, 135.9, 150.0, 165.6. LCMS (ESI⁺): 530.25 *m/z* [M + H]⁺. Purity = 99.4%.

Diethyl [1-(3,4-dichlorophenyl)-3-[N-(4-phenylbutoxy)acetamido]propyl] phosphonate (14g). Slightly yellow oil (0.033 g, 16%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.16 (t, 3H), 1.28 (t, 3H), 1.66 (dq, 5H), 2.02 (s, 3H), 2.16 (m, 1H), 2.40 (1H), 2.56–2.69 (m, 2H), 2.90–3.09 (m, 1H), 3.38–3.57 (m, 2H), 3.62–3.76 (m, 2H), 3.77–3.89 (m, 1H), 3.90–3.98 (m, 1H), 3.99–4.19 (m, 1H), 7.05–7.50 (m, 9H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.3, 16.4, 26.8, 27.6, 27.9, 35.6, 62.3, 62.8, 74.0, 125.9, 128.4, 128.4, 128.6, 128.7, 130.4, 131.1, 131.1. LCMS (ESI⁺): 530.25 *m/z* [M + H]⁺. Purity = 94.6%.

Diethyl [3-[N-[[4-bromophenyl]methoxy]acetamido]-1-(3,4-dichlorophenyl)propyl] phosphonate (14h). Slightly yellow oil (0.470 g, 70%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.06–1.41 (m, 6H), 1.59 (s, 1H), 2.02 (m, 3H), 2.17 (m, 1H), 2.43 (m, 1H), 2.91–3.10 (m, 1H), 3.32–3.64 (m, 2H), 3.75–4.20 (m, 4H), 4.65 (s, 2H), 7.02–7.60 (m, 7H). LCMS (ESI⁺): 568.25 *m/z* [M + 2 + H]⁺. Purity = 97.4%.

Diethyl [1-(3,4-dichlorophenyl)-3-[N-[[4'-(propan-2-yl)-[1,1'-biphenyl]-4-yl]methoxy]acetamido]propyl]phosphonate (14i). Compound **14h** (0.310 g, 0.547 mmol) was dissolved in toluene (2.8 mL). To the solution was added Pd(PPh₃)₄ (0.064 g, 0.055 mmol), and the reaction was stirred at room temperature for 15 minutes. After 15 minutes, a solution of 4-isopropylphenyl boronic acid (0.448 g, 2.73 mmol) in ethanol (1 mL) was added to the reaction mixture, and it stirred for 15 minutes. Then, 0.83 mL 2 M Na₂CO₃ was added. The reaction mixture was stirred at 75 °C overnight. The mixture was filtered with a membrane filter, then the toluene was removed under reduced pressure. The crude residue was dissolved in dichloromethane and washed with distilled water (20 mL). The aqueous phase was extracted with dichloromethane (3 × 20 mL). The organic phase was dried over sodium sulfate and filtered. Dichloromethane was removed under reduced pressure and the crude product was purified *via* column (5:1 dichloromethane:ethyl acetate) to afford a yellow oil (0.065 g, 19%). ¹H NMR (400 MHz, CDCl₃)

δ (ppm): 1.14–1.28 (m, 12H), 1.58 (t, 2H), 2.00 (s, 3H), 2.45 (m, 1H), 2.97 (m, 2H), 3.48 (m, 2H), 3.79–4.10 (m, 3H), 4.79 (t, 2H), 7.09–7.73 (m, 11H). ¹³C NMR (101 MHz, CDCl₃) δ (ppm): 16.3, 23.8, 23.9, 33.8, 59.9, 113.9, 126.9, 127.0, 127.3, 128.4, 128.6, 129.6, 131.1, 132.0, 132.1, 133.8. LCMS (ESI⁺): 606.25 *m/z* [M + H]⁺. Purity = 93.9%.

Diethyl [3-{acetyl[1-(4-chlorophenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (14j). Slightly yellow oil (0.153 g, 66%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.15 (t, 3H), 1.28 (t, 3H), 1.51 (s, 3H), 1.94 (s, 1H), 1.99 (s, 3H), 2.05 (s, 1H), 2.24–2.37 (m, 1H), 2.74 (s, 1H), 2.80–2.98 (m, 2H), 3.45–3.57 (m, 1H), 3.75–3.82 (m, 1H), 3.90–3.95 (m, 1H), 4.04 (bs, 2H), 4.86 (q, 1H), 7.09 (t, 1H), 7.81 (d, 1H), 7.31 (s, 2H), 7.37–7.41 (m, 2H). LCMS (ESI⁺): 535.25 *m/z* [M + 2 + H]⁺. Purity = 97.9%.

Diethyl [3-{acetyl[1-(4-methylphenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (14k). Slightly yellow oil (0.100 g, 34%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.14 (t, 3H), 1.28 (t, 3H), 1.50 (d, 3H), 2.02 (s, 3H), 1.91 (s, 1H), 2.78–3.01 (m, 1H), 3.39–3.59 (m, 1H), 3.75–3.87 (m, 1H), 3.98–4.11 (m, 2H), 4.56–4.79 (m, 1H), 6.73–6.89 (m, 2H), 7.06–7.18 (m, 3H), 7.31–7.44 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.5, 19.4, 29.6, 38.6, 68.1, 82.5, 85.5, 120.7, 131.1, 131.4, 143.5, 143.3, 147.3, 155.9, 166.4. LCMS (ESI⁺): 516.25 *m/z* [M + H]⁺. Purity = 96.5%.

Diethyl [3-{acetyl[1-(4-methoxyphenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (14l). Slightly yellow oil (0.058 g, 21%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.14 (t, 3H), 1.28 (t, 3H), 1.50 (d, 3H), 2.02 (s, 3H), 1.91 (s, 3H), 2.30 (m, 1H), 3.39–3.59 (m, 1H), 3.75–3.87 (d, 4H), 3.88–3.97 (m, 1H), 3.98–4.11 (m, 2H), 4.56–4.79 (m, 1H), 6.73–6.89 (m, 2H), 7.06–7.18 (m, 3H), 7.31–7.44 (m, 2H). LCMS (ESI⁺): 532.25 *m/z* [M + H]⁺. Purity = 97.5%.

Diethyl [3-{acetyl[1-(4-trifluoromethylphenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (14m). Slightly yellow oil (0.083 g, 36%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.14 (s, 3H), 1.27 (s, 3H), 1.53 (s, 3H), 1.94 (s, 1H), 2.00 (s, 3H), 2.04–2.10 (m, 1H), 2.33 (bs, 1H), 2.74 (s, 1H), 2.80 (bs, 1H), 2.87–3.04 (m, 1H), 3.54 (bs, 1H), 3.81 (bs, 1H), 3.92 (bs, 1H), 4.04 (bs, 2H), 4.78 (q, 1H), 7.10 (t, 1H), 7.38 (s, 4H), 7.55–7.61 (d, 2H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.2, 22.4, 24.7, 46.7, 68.8, 102.4, 110.5, 125.7, 127.7, 133.6, 135.1, 136.9, 162.7, 167.4. LCMS (ESI⁺): 570.25 *m/z* [M + H]⁺. Purity = 98.7%.

Diethyl [3-{acetyl[1-(4-fluorophenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (14n). Slightly yellow oil (0.050 g, 37%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.16 (t, 3H), 1.30 (t, 3H), 1.51 (s, 3H), 1.96 (s, 1H), 1.99 (s, 3H), 2.05 (s, 1H), 2.25–2.39 (m, 1H), 2.74 (s, 1H), 2.81–2.99 (m, 2H), 3.47–3.59 (m, 1H), 3.77–3.83 (m, 1H), 3.92–3.96 (m, 1H), 4.04 (bs, 2H), 4.86 (q, 1H), 7.10 (t, 1H), 7.83 (d, 1H), 7.33 (s, 2H), 7.37–7.41 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 16.3, 20.6, 26.2, 40.8, 42.2, 45.2, 62.3, 81.9, 115.5, 128.9, 130.4, 130.9, 131.1, 131.4, 132.5, 135.8, 161.6, 164.1. LCMS (ESI⁺): 520.25 *m/z* [M + H]⁺. Purity = 96.8%.



General procedure for synthesis of diammonium salt compounds 6a–n

A solution of **13**, **14b–n** (1 eq.) in anhydrous dichloromethane (0.1 M) under nitrogen was cooled to 0 °C. Trimethylsilyl bromide (3–5 eq.) was added dropwise to the solution. The mixture was warmed to room temperature and stirred overnight. Trimethylsilyl bromide and dichloromethane were removed under reduced pressure. The crude residue was stirred at room temperature in anhydrous methanol for 1–2 hours. The methanol was removed under reduced pressure. 7 M NH₃ in methanol (2 eq.) was added to the mixture, and it was allowed to stir at room temperature for 1–2 hours. The excess methanol was removed under reduced pressure to yield the pure salts.

Diammonium [1-(3,4-dichlorophenyl)-3-(N-hydroxyacetamido)propyl] phosphonate (6a). Off-white solid (0.074 g, 57%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 2.05 (s, 3H), 3.10–3.22 (m, 2H), 3.58 (bs, 1H), 3.74–4.09 (m, 1H), 4.40–4.60 (m, 1H), 7.94 (m, 4H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 11.6, 14.9, 42.0, 42.8, 52.5, 115.8, 120.2, 122.7, 124.1, 129.4, 174.1. LCMS (ESI⁺): 341.25 *m/z* [M + H]⁺. HRMS (ESI⁺) calculated for C₉H₁₃Cl₂NO₄P 299.9959, found 299.9951 [M–2NH₃–COCH₃ + H]⁺. Purity = 99.7%.

Diammonium {3-[N-(benzyloxy)acetamido]-1-(3,4-dichlorophenyl)propyl} phosphonate (6b). Off-white solid (0.017 g, quantitative). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 2.00 (s, 3H), 2.41 (m, 1H), 2.75–2.81 (m, 1H), 3.27–3.58 (m, 3H), 4.79 (bs, 2H), 6.68–6.89 (m, 4H), 7.28–7.46 (m, 4H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 19.8, 26.1, 42.3, 43.8, 74.5, 126.0, 129.1, 129.8, 130.0, 130.2, 131.5, 132.1, 133.4, 136.1, 136.7, 172.3. HRMS (ESI[–]) calculated for C₁₈H₁₉Cl₂NO₅P 430.0378, found 430.0383 [M–2NH₃–H][–]. Purity = 95.0%.

Diammonium [1-(3,4-dichlorophenyl)-3-{N-[(naphthalen-2-yl)methoxy]acetamido}propyl]phosphonate (6c). Orange solid (0.107 g, 98%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 2.01 (s, 3H), 2.41 (m, 1H), 2.55 (m, 1H), 2.72–2.87 (m, 1H), 3.19–3.41 (m, 1H), 3.47–3.66 (m, 1H), 4.84–5.06 (m, 2H), 7.22 (d, 2H), 7.44–7.51 (m, 3H), 7.57–7.59 (m, 3H), 7.94 (m, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 20.4, 27.6, 42.6, 43.9, 75.6, 126.4, 126.5, 127.6, 127.9, 128.0, 128.4, 129.4, 129.7, 130.3, 130.8, 132.4, 132.7, 132.8, 141.7, 175.7. HRMS (ESI⁺) calculated for C₂₂H₂₃Cl₂NO₅P 482.0691, found 482.0693 [M–2NH₃ + H]⁺. Purity = 98.4%.

Diammonium {3-[N-({1,1'-biphenyl}-4-yl)methoxy]acetamido]-1-(3,4-dichlorophenyl)propyl} phosphonate (6d). Off-white solid (0.041 g, 89%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.97 (s, 3H), 2.32 (m, 1H), 2.56–2.66 (m, 1H), 3.27–3.58 (m, 3H), 4.72–4.89 (m, 2H), 7.16 (d, 2H), 7.36–7.38 (m, 1H), 7.40–7.41 (m, 1H), 7.42–7.45 (m, 2H), 7.47–7.49 (m, 1H), 7.66 (t, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 20.8, 21.6, 68.5, 75.5, 76.0, 126.8, 126.9, 127.3, 128.0, 128.4, 128.5, 128.8, 129.8, 130.1, 130.7, 131.2, 132.8, 133.1, 133.2, 134.3, 142.1, 176.0. HRMS (ESI[–]) calculated for C₂₄H₂₃Cl₂NO₅P 506.0691, found 506.0677 [M–2NH₃–H][–]. Purity = 96.2%.

Diammonium [1-(3,4-dichlorophenyl)-3-{N-(1-phenylethoxy)acetamido}propyl] phosphonate (6e). Orange solid (46 mg, quantitative). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 0.78 (d, 3H), 1.73 (d, 2H), 2.43 (s, 3H), 2.98 (m, 1H), 3.17 (m, 1H), 3.26–3.51 (m, 1H), 4.66–4.84 (m, 1H), 6.98–7.79 (m, 8H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 22.5, 23.3, 28.4, 29.8, 38.1, 41.2, 67.4, 127.3, 128.2, 128.4, 128.6, 128.7, 129.5, 130.1, 131.6, 131.7, 135.5, 140.3, 167.0. HRMS (ESI[–]) calculated for C₁₉H₂₁Cl₂NO₅P 444.0534, found 444.0530 [M–2NH₃–H][–]. Purity = 95.2%.

Diammonium [1-(3,4-dichlorophenyl)-3-{N-([4-(propan-2-yl)phenyl]methoxy)acetamido}propyl] phosphonate (6f). Slightly yellow solid (0.065 g, 97%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.18 (s, 6H), 1.90 (s, 3H), 2.24–2.37 (m, 1), 2.74–2.85 (m, 2H), 3.20 (bs, 1H), 3.49 (bs, 1H), 4.59–4.79 (m, 2H), 6.91 (bs, 1H), 7.23 (s, 4H), 7.41–7.48 (m, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 20.3, 23.8, 27.4, 33.2, 42.3, 43.6, 75.4, 126.3, 128.2, 129.4, 129.7, 130.0, 130.3, 130.6, 132.1, 141.1, 149.0, 171.1. HRMS (ESI⁺) calculated for C₂₁H₂₇Cl₂NO₅P 474.1004, found 474.1005 [M–2NH₃ + H]⁺. Purity = 95.4%.

Diammonium [1-(3,4-dichlorophenyl)-3-{N-(4-phenylbutoxy)acetamido}propyl] phosphonate (6g). Off-white solid (0.027 g, 98%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.17–1.31 (m, 4H), 2.09 (m, 3H), 2.64 (m, 2H), 3.01–3.07 (m, 2H), 3.55 (m, 2H), 3.61–3.83 (m, 2H), 7.16–7.55 (m, 8H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 20.4, 26.9, 27.2, 27.7, 35.2, 42.6, 43.9, 73.6, 125.5, 127.9, 128.1, 128.6, 128.9, 129.8, 131.0, 131.1, 132.2, 135.8, 136.8, 141.9, 171.7. HRMS (ESI[–]) calculated for C₂₁H₂₅Cl₂NO₅P 472.0847, found 472.0845 [M–2NH₃–H][–]. Purity = 95.7%.

Diammonium {3-[N-([4-bromophenyl]methoxy)acetamido]-1-(3,4-dichlorophenyl)propyl} phosphonate (6h). Light orange solid (0.045 g, quantitative). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.95 (s, 3H), 2.27 (bs, 1H), 2.74–2.87 (m, 1H), 3.03 (bs, 1H), 3.19–3.31 (m, 1H), 3.48–3.72 (m, 2H), 4.69–4.82 (m, 2H), 7.17–7.32 (m, 3H), 7.48–7.56 (m, 4H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 20.3, 29.0, 42.8, 43.6, 74.6, 121.9, 128.2, 129.5, 130.2, 130.7, 131.3, 131.8, 134.1, 139.9, 141.5, 171.7. HRMS (ESI[–]) calculated for C₁₈H₁₈BrCl₂NO₅P 507.9483, found 507.9485 [M–2NH₃–H][–]. Purity = 98.7%.

Diammonium [1-(3,4-dichlorophenyl)-3-{N-([4'-(propan-2-yl)-[1,1'-biphenyl]-4-yl]methoxy)acetamido}propyl]phosphonate (6i). Tan solid (0.020 g, quantitative). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.25 (s, 6H), 1.99 (s, 3H), 2.22 (bs, 1H), 2.29 (bs, 1H), 2.70 (bs, 1H), 2.92 (m, 1H), 3.15–3.26 (m, 2H), 4.70–4.88 (m, 2H), 6.52 (bs, 4H), 7.16–7.41 (m, 4H), 7.48–7.69 (m, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 21.0, 23.9, 28.7, 35.4, 43.8, 45.1, 75.7, 117.9, 118.4, 120.6, 121.6, 122.6, 125.5, 126.7, 128.7, 130.2, 131.9, 137.6, 138.2, 138.8, 142.2, 172.3. HRMS (ESI[–]) calculated for C₂₇H₂₉Cl₂NO₅P 548.1160, found 548.1163 [M–2NH₃–H][–]. Purity = 96.5%.

Diammonium [3-{acetyl[1-(4-chlorophenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (6j). Off-white solid (0.105 g, quantitative). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.10 (d, 3H), 1.93 (s, 3H), 2.22–2.24 (m, 2H), 2.66–2.73 (m, 1H), 3.40 (bs, 2H), 4.88 (bs, 1H), 7.21–7.54 (m, 7H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 19.9, 20.5,



26.8, 42.3, 43.8, 80.7, 127.2, 128.4, 129.1, 129.7, 130.3, 130.6, 133.0, 139.4, 140.9, 143.1, 170.5. HRMS (ESI[−]) calculated for C₁₉H₂₀Cl₃NO₅P 478.0145, found 478.0150 [M-2NH₃-H][−]. Purity = 97.6%.

Diammonium [3-{acetyl[1-(4-methylphenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (6k). Off-white solid (0.095 g, quantitative). ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 1.40 (d, 3H), 1.88 (s, 3H), 2.10 (bs, 1H), 2.25 (s, 3H), 2.41–2.46 (m, 1H), 3.03–3.26 (m, 1H), 3.40 (s, 2H), 4.75 (q, 1H), 7.00 (d, 1H), 7.10–7.14 (m, 3H), 7.17 (d, 1H), 7.35 (s, 1H), 7.40 (d, 1H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 19.9, 20.5, 20.8, 27.7, 44.1, 45.5, 81.3, 127.2, 127.4, 129.3, 130.0, 130.4, 137.3, 137.5, 137.7, 143.0, 172.1. HRMS (ESI[−]) calculated for C₂₀H₂₃Cl₂NO₅P 458.0691, found 458.0691 [M-2NH₃-H][−]. Purity = 98.6%.

Diammonium [3-{acetyl[1-(4-methoxyphenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (6l). Off-white solid (0.045 mg, quantitative). ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 1.28 (d, 3H), 1.93 (s, 3H), 2.38–2.44 (m, 1H), 2.63–2.66 (m, 1H), 2.97 (bs, 1H), 3.17 (m, 2H), 3.74 (s, 3H), 4.66–4.84 (m, 1H), 6.85–6.88 (m, 1H), 7.16 (d, 2H), 7.42 (d, 3H), 7.67–7.73 (m, 1H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 20.2, 21.3, 28.2, 42.3, 46.4, 52.8, 77.1, 114.9, 126.5, 126.9, 129.2, 130.4, 132.0, 135.2, 136.4, 139.2, 164.4, 174.7. HRMS (ESI[−]) calculated for C₂₀H₂₃Cl₂NO₆P 474.0640, found 474.0640 [M-2NH₃-H][−]. Purity = 96.4%.

Diammonium [3-{acetyl[1-(4-trifluoromethylphenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (6m). Off-white solid (0.077 g, quantitative). ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 1.36 (d, 3H), 2.00 (s, 3H), 2.73–2.79 (m, 1H), 3.05 (bs, 1H), 3.28–3.35 (m, 1H), 3.50 (bs, 1H), 3.56–3.66 (m, 1H), 3.81 (m, 1H), 4.21 (m, 1H), 6.94 (m, 1H), 7.26 (m, 2H), 7.51 (m, 2H), 7.78 (m, 1H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 20.5, 22.4, 28.9, 43.5, 46.1, 78.8, 123.4, 124.6, 125.3, 127.4, 129.5, 132.7, 133.8, 135.1, 136.1, 142.2, 168.2. HRMS (ESI[−]) calculated for C₂₀H₂₀Cl₂F₃NO₅P 512.0408, found 512.0414 [M-2NH₃-H][−]. Purity = 97.8%.

Diammonium [3-{acetyl[1-(4-fluorophenyl)ethoxy]amino}-1-(3,4-dichlorophenyl)propyl] phosphonate (6n). Off-white solid (0.037 g, quantitative). ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 1.10 (d, 3H), 1.93 (s, 3H), 2.22 (bs, 2H), 2.66 (bs, 1H), 2.74 (bs, 1H), 3.40 (bs, 2H), 4.88 (bs, 1H), 6.97 (m, 1H), 7.03–7.62 (m, 2H), 7.42–7.35 (m, 1H), 7.62–7.74 (m, 3H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 19.2, 19.7, 27.1, 42.3, 44.3, 75.3, 117.8, 126.4, 127.8, 130.4, 130.7, 132.1, 135.1, 136.3, 137.6, 161.0, 173.1. HRMS (ESI[−]) calculated for C₁₉H₂₀Cl₂FNO₅P 462.0440, found 462.0442 [M-2NH₃-H][−]. Purity = 95.4%.

General procedure for synthesis of dipivaloyloxymethylene prodrugs 7c–g,i

Salts **6c–g,i** (1 eq.) were dissolved in anhydrous *N,N*-dimethylformamide (0.01 M). Triethyl amine (6 eq.) and pivaloyloxymethyl chloride (6 eq.) were added to the solution at room temperature. The reaction mixture was heated to 60

°C and was stirred overnight. The reaction mixture was extracted with water (3 × 50 mL) and diethyl ether (3 × 50 mL). Excess ether was removed under reduced pressure. The crude residue was dissolved in hexanes (50 mL) and washed with water (3 × 50 mL). The organic layer was dried over sodium sulfate and filtered. The hexanes was removed under reduced pressure and the residue was purified by normal phase silica column chromatography with 60:40 hexanes: ethyl acetate.

([1-(3,4-Dichlorophenyl)-3-{N-[(naphthalen-2-yl)methoxy]acetamido}propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl)oxy)methyl-2,2-dimethylpropanoate (7c). Colorless oil was obtained as the product (0.036 g, 17%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.08–1.30 (m, 18H), 1.64 (s, 1H), 2.04 (t, 3H), 2.42 (m, 1H), 3.08 (m, 1H), 3.51 (m, 2H), 4.85 (s, 2H), 5.45–5.63 (m, 4H), 7.07–7.85 (m, 10H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.5, 26.3, 26.7, 38.6, 39.0, 42.2, 76.9, 81.7, 126.3, 126.6, 127.8, 128.1, 128.6, 128.8, 130.6, 131.1, 131.2, 131.6, 131.9, 132.8, 133.1, 133.4, 134.4, 135.5, 161.1, 176.8. HRMS (ESI⁺) calculated for C₃₄H₄₃Cl₂NO₉P 710.2052, found 710.2073 [M + H]⁺. Purity = 96.5%.

([3-{N-[(1,1'-Biphenyl)-4-yl]methoxy]acetamido}-1-(3,4-dichlorophenyl)propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl)oxy)methyl-2,2-dimethylpropanoate (7d). Colorless oil (0.038 g, 25%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.16 (s, 9H), 1.19 (s, 9H), 2.03 (s, 3H), 2.33 (m, 1H), 3.17 (m, 1H), 3.44 (m, 2H), 4.74 (2H), 5.41 (m, 4H), 7.22–7.53 (m, 12H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.5, 26.7, 26.8, 38.7, 40.9, 41.5, 42.3, 76.3, 81.7, 127.1, 127.5, 127.7, 128.7, 128.8, 128.9, 129.7, 130.6, 131.1, 131.2, 134.5, 140.3, 140.0, 173.1, 176.8. HRMS (ESI⁺) calculated for C₃₆H₄₅Cl₂NO₉P 736.2209, found 736.2192 [M + H]⁺. Purity = 95.3%.

([1-(3,4-Dichlorophenyl)-3-{N-(1-phenylethoxy)acetamido}propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl)oxy)methyl-2,2-dimethylpropanoate (7e). Colorless oil (0.028 g, 17%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.16–1.21 (m, 21H), 1.51 (m, 3H), 1.89 (t, 3H), 2.99 (m, 1H), 4.66 (1H), 5.29–5.63 (m, 5H), 7.04–7.37 (m, 8H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.2, 20.3, 25.6, 26.8, 26.7, 38.7, 41.4, 44.7, 75.4, 81.6, 127.1, 127.2, 127.6, 128.5, 128.7, 130.5, 131.0, 131.2, 133.6, 134.3, 144.9, 167.8, 176.8. HRMS (ESI⁺) calculated for C₃₁H₄₃Cl₂NO₉P 674.2052, found 674.2053 [M + H]⁺. Purity = 97.7%.

([1-(3,4-Dichlorophenyl)-3-{N-[4-(propan-2-yl)phenyl]methoxy]acetamido}propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl)oxy)methyl-2,2-dimethylpropanoate (7f). Colorless oil (0.062 g, 32%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.18 (d, 18H), 1.25 (d, 6H), 1.99 (s, 3H), 2.38 (1H), 2.86 (m, 1H), 3.09 (m, 1H), 3.45 (m, 2H), 4.68 (s, 2H), 5.50–5.66 (m, 4H), 7.12–7.37 (m, 7H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.3, 23.9, 26.2, 26.7, 38.7, 40.8, 43.3, 76.1, 81.7, 126.8, 128.7, 129.4, 130.6, 131.6, 131.4, 131.9, 132.7, 134.4, 134.5, 150.0, 171.6, 176.9. HRMS (ESI⁺) calculated for C₃₃H₄₇Cl₂NO₉P 702.2365, found 702.2360 [M + H]⁺. Purity = 98.7%.

([1-(3,4-Dichlorophenyl)-3-{N-(4-phenylbutoxy)acetamido}propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl)oxy)methyl-2,2-dimethylpropanoate (7g). Colorless oil (0.026



g, 13%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.16–1.27 (m, 18H), 1.61–1.79 (m, 4H), 2.05 (s, 3H), 2.23 (m, 1H), 2.43 (m, 1H), 2.69 (m, 2H), 3.24 (m, 1H), 3.55 (m, 2H), 3.77 (m, 2H), 5.52–5.70 (m, 4H), 7.20–7.455 (m, 8H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 20.1, 26.3, 26.8, 27.6, 27.8, 29.7, 35.6, 38.7, 40.9, 42.3, 42.8, 74.2, 81.7, 126.0, 128.4, 128.5, 128.7, 130.6, 131.2, 132.0, 134.5, 141.7, 165.8, 176.8. HRMS (ESI^+) calculated for $\text{C}_{33}\text{H}_{46}\text{Cl}_2\text{NO}_9\text{P}$ 701.2287, found 702.2363 $[\text{M} + \text{H}]^+$. Purity = 97.7%.

(((1-(3,4-Dichlorophenyl)-3-(N-([4'-(propan-2-yl)-[1,1'-biphenyl]-4-yl]methoxy)acetamido)propyl)({((2,2-dimethylpropanoyl)oxy)methoxy})phosphoryl)oxy)methyl 2,2-dimethylpropanoate (7i). Colorless oil (0.036 g, 26%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.16–1.30 (m, 24H), 1.70 (s, 1H), 2.03 (s, 3H), 2.25 (m, 1H), 2.43 (m, 1H), 3.16 (m, 1H), 3.49 (m, 2H), 4.73 (s, 2H), 5.47–5.64 (m, 4H), 7.11–7.59 (m, 11H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 24.0, 26.7, 26.8, 29.7, 33.8, 38.7, 81.8, 126.9, 127.0, 127.3, 129.6, 130.6. HRMS (ESI^+) calculated for $\text{C}_{39}\text{H}_{51}\text{Cl}_2\text{NO}_9\text{P}$ 778.2678, found 778.2650 $[\text{M} + \text{H}]^+$. Purity = 95.6%.

General procedure for synthesis of dipivaloyloxymethylene prodrugs 7h,j–k

To a solution of salts **6h,j–l** (1 eq.) in anhydrous tetrahydrofuran (0.1 M) in a nitrogen atmosphere were added *N,N*-diisopropylethylamine (6 eq.) and pivaloyloxymethyl chloride (6 eq.) at room temperature. The reaction mixture was heated to 60 °C and stirred for 48–72 hours. The tetrahydrofuran was removed under reduced pressure. The crude residue was dissolved in dichloromethane (20 mL) and washed with saturated NaHCO_3 (3 $\times$ 10 mL), followed by brine (1 $\times$ 15 mL). The organic phase was dried over anhydrous sodium sulfate and filtered. The dichloromethane was removed under reduced pressure, and the residue was purified *via* column chromatography (5 : 1 dichloromethane : ethyl acetate) to yield the desired compound.

(((3-(N-([4-Bromophenyl)methoxy]acetamido)-1-(3,4-dichlorophenyl)propyl)({((2,2-dimethylpropanoyl)oxy)methoxy})phosphoryl)oxy)methyl 2,2-dimethylpropanoate (7h). A slightly yellow oil (0.090 g, 49%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.10 (s, 18H), 1.58 (bs, 1H), 1.19 (s, 3H), 2.08 (bs, 1H), 2.31 (bs, 1H), 3.00–3.08 (m, 1H), 3.36–3.43 (m, 1H), 4.58 (bs, 1H), 5.42–5.53 (m, 4H), 7.08 (s, 3H), 7.18 (s, 1H), 7.28 (s, 2H), 7.42 (s, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 20.4, 26.3, 26.8, 38.7, 40.8, 42.2, 43.6, 75.8, 81.8, 123.3, 128.6, 130.6, 130.7, 131.1, 132.0, 132.8, 133.1, 134.4, 172.3, 176.7. HRMS (ESI^+) calculated for $\text{C}_{30}\text{H}_{39}\text{BrCl}_2\text{NO}_9\text{P}$ 737.0923, found 736.0845 $[\text{M}-\text{H}]^-$. Purity = 96.2%.

(((1-(3,4-Dichlorophenyl)-3-(N-([1-(4-chlorophenyl)ethoxy]acetamido)propyl)({((2,2-dimethylpropanoyl)oxy)methoxy})phosphoryl)oxy)methyl 2,2-dimethylpropanoate (7j). A slightly yellow oil (0.111 g, 13%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.14 (s, 18H), 1.42 (d, 3H), 1.89 (m, 3H), 2.22 (s, 1H), 2.66 (s, 1H), 2.89–2.95 (m, 1H), 3.43 (bs, 1H), 4.64 (bs, 1H), 5.58–5.42 (m, 4H), 7.00–7.32 (m, 7H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 20.3, 20.6, 25.6, 26.8, 29.7, 38.7, 40.9, 44.9, 77.9, 81.8,

126.1, 128.6, 130.6, 131.1, 132.0, 132.7, 134.4, 134.7, 138.5, 143.1, 173.3, 176.8. HRMS (ESI^-) calculated for $\text{C}_{31}\text{H}_{40}\text{Cl}_3\text{NO}_9\text{P}$ 706.1506, found 706.1509 $[\text{M}-\text{H}]^-$. Purity = 97.6%.

(((1-(3,4-Dichlorophenyl)-3-(N-([1-(4-methylphenyl)ethoxy]acetamido)propyl)({((2,2-dimethylpropanoyl)oxy)methoxy})phosphoryl)oxy)methyl 2,2-dimethylpropanoate (7k). A slightly yellow oil (0.064 g, 19%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.10 (s, 9H), 1.15 (s, 9H), 1.43 (s, 3H), 1.82 (s, 1H), 1.89 (s, 3H), 2.25 (s, 3H), 2.28 (s, 1H), 2.52–2.65 (m, 1H), 2.79–3.02 (m, 2H), 3.40 (bs, 1H), 4.57 (bs, 1H), 5.39–5.63 (m, 4H), 7.04–7.13 (m, 1H), 7.20–7.26 (m, 1H), 7.28–7.38 (m, 5H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 20.2, 20.5, 21.2, 25.7, 26.7, 38.6, 42.3, 44.7, 81.7, 127.1, 128.2, 128.7, 129.1, 129.3, 130.5, 131.8, 132.7, 134.4, 136.9, 138.8, 168.1, 176.8. HRMS (ESI^-) calculated for $\text{C}_{32}\text{H}_{43}\text{Cl}_2\text{NO}_9\text{P}$ 686.2052, found 686.2055 $[\text{M}-\text{H}]^-$. Purity = 98.3%.

(((1-(3,4-Dichlorophenyl)-3-(N-([1-(4-methoxyphenyl)ethoxy]acetamido)propyl)({((2,2-dimethylpropanoyl)oxy)methoxy})phosphoryl)oxy)methyl 2,2-dimethylpropanoate (7l). Compound **12** (1 eq.) was dissolved in anhydrous tetrahydrofuran (0.1 M) under a nitrogen atmosphere and cooled to 0 °C. Sodium hydride (1.2 eq.) was added to the solution, and the reaction mixture stirred for 20 minutes. To the reaction mixture was added 1-(1-bromoethyl)-4-methoxybenzene (2 eq.). The mixture was warmed to room temperature and stirred for 48 hours. After removing the tetrahydrofuran under reduced pressure, the residue was dissolved in dichloromethane and quenched with water. The aqueous phase was extracted with dichloromethane (5 $\times$ 20 mL). The organic phase was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure. The crude product was purified *via* column chromatography (97 : 3 dichloromethane : methanol). Slightly yellow oil (0.010 g, 4%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.18 (s, 9H), 1.22 (s, 9H), 1.85 (s, 3H), 2.09–2.19 (m, 1H), 2.65–2.39 (m, 1H), 3.14–3.28 (m, 1H), 3.44–3.70 (m, 2H), 3.65 (s, 3H), 3.77–3.89 (m, 1H), 5.46–5.66 (m, 4H), 6.94 (bs, 1H), 7.15 (d, 1H), 7.26 (s, 2H), 7.38–7.43 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 20.2, 20.7, 26.9, 27.3, 38.9, 40.2, 46.2, 55.3, 113.8, 126.3, 126.7, 129.0, 130.1, 132.0, 134.4, 135.4, 139.2, 159.1, 169.1, 175.2. HRMS (ESI^+) calculated for $\text{C}_{32}\text{H}_{45}\text{Cl}_2\text{NO}_{10}\text{P}$ 704.2158, found 704.1966 $[\text{M} + \text{H}]^+$. Purity = 97.2%.

General procedure for synthesis of dipivaloyloxymethylene prodrugs 7m,n

A solution of **14m,n** (1 eq.) in anhydrous dichloromethane (0.1 M) under nitrogen was cooled to 0 °C. Trimethylsilyl bromide (3 eq.) was added dropwise to the solution under nitrogen. The mixture was warmed to room temperature and stirred overnight. Trimethylsilyl bromide and dichloromethane were removed under reduced pressure. The crude residue was stirred at room temperature in anhydrous methanol for one to two hours. The methanol was removed under reduced pressure. To a solution of the crude residue in tetrahydrofuran (0.1 M) were added *N,N*-



diisopropylethylamine (6 eq.) and pivaloyloxymethyl chloride (6 eq.) at room temperature. The reaction mixture was heated to 60 °C and stirred for 48–72 hours. The tetrahydrofuran was removed under reduced pressure. The crude residue was dissolved in dichloromethane (20 mL) and washed with saturated NaHCO₃ (3 × 10 mL), followed by brine (1 × 15 mL). The organic phase was dried over anhydrous sodium sulfate and filtered. The dichloromethane was removed under reduced pressure, and the residue was purified *via* column chromatography (97:3 dichloromethane:methanol) to yield the desired compound.

[[[1-(3,4-Dichlorophenyl)-3-{N-[1-(4-trifluoromethylphenyl)ethoxy]acetamido}propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl]oxy)methyl 2,2-dimethylpropanoate (7m). Slightly yellow oil (0.0091 g, 9%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.10 (s, 9H), 1.14 (s, 9H), 1.43 (s, 3H), 1.83 (s, 1H), 1.87 (s, 3H), 2.19 (s, 1H), 2.67 (s, 1H), 2.85–2.99 (m, 2H), 3.43 (bs, 1H), 5.37–5.57 (m, 4H), 6.89–7.00 (m, 3H), 7.13 (m, 2H), 7.21–7.33 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 23.0, 23.8, 25.6, 26.7, 26.8, 28.9, 29.7, 30.4, 38.8, 81.8, 82.2, 125.6, 125.9, 127.4, 128.8, 130.3, 130.6, 131.2, 134.3, 145.6, 172.0, 176.8. HRMS (ESI[−]) calculated for C₃₂H₄₀Cl₂F₃NO₉P 740.1770, found 740.1771 [M−H][−]. Purity = 98.4%.

[[[1-(3,4-Dichlorophenyl)-3-{N-[1-(4-fluorophenyl)ethoxy]acetamido}propyl]([[(2,2-dimethylpropanoyl)oxy]methoxy})phosphoryl]oxy)methyl 2,2-dimethylpropanoate (7n). Slight yellow oil (0.100 g, 15%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 1.12 (s, 9H), 1.16 (s, 9H), 1.38 (d, 3H), 1.97 (m, 3H), 2.13 (s, 1H), 2.37 (s, 1H), 3.01–3.14 (m, 2H), 3.33–3.51 (m, 1H), 4.61–4.71 (m, 1H), 5.39–5.63 (m, 4H), 7.04–7.13 (m, 1H), 7.20–7.26 (m, 2H), 7.28–7.38 (m, 4H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 20.1, 20.4, 25.6, 26.8, 29.6, 38.6, 42.2, 44.8, 81.7, 115.4, 128.6, 128.9, 130.5, 130.9, 131.1, 132.6, 135.6, 135.8, 161.6, 173.1, 176.7. HRMS (ESI⁺) calculated for C₃₁H₄₂Cl₂FNO₉P 692.1958, found 692.1957 [M + H]⁺. Purity = 96.8%.

P. falciparum and *M. tuberculosis* dxr cloning, expression, and purification

The *P. falciparum* and *M. tuberculosis* dxr genes were cloned, expressed, and purified as previously described.^{37,60} Briefly, the *Pf* and *Mtb* dxr genes were each cloned into a pET101/DTOP vector. The recombinant plasmid was transformed into chemically competent *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene, LA Jolla, CA). For protein expression, a 10 mL overnight seed culture of *E. coli* BL21 CodonPlus (DE3)-RIL + *MtbDXR* (or *PfDXR*) was added to 1 L of LB media and incubated with shaking at 37 °C and 250 rpm. Once an OD₆₀₀ of 1.8 was achieved, protein expression was induced using 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the culture was allowed to incubate for an additional 18 h. Cells were harvested *via* centrifugation (4650 × *g*, 20 min) and stored at −80 °C. Protein was isolated and purified from the cells *via* chemical lysis and affinity chromatography. Cell lysis was achieved using lysis buffer A (100 mM Tris pH 8, 0.032% lysozyme, 3 mL per mg cell pellet), followed by lysis

buffer B (0.1 M CaCl₂, 0.1 M MgCl₂, 0.1 M NaCl, 0.020% DNASE, 3 mL per mg cell pellet). Centrifugation (48 000 × *g*, 20 min) yielded the clarified cell lysate that was passed through a TALON immobilized metal affinity column (Clontech Laboratories, Mountain View, CA). The column was washed with 20 column volumes of 1× equilibrium buffer (50 mM HEPES pH 7.5, 300 mM NaCl), 10 column volumes of 1× wash buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole), and 15 column volumes of 2× wash buffer (100 mM HEPES pH 7.5, 600 mM NaCl, 20 mM imidazole). The protein was then eluted with 5 column volumes of 1× elution buffer (150 mM imidazole pH 7.0, 300 mM NaCl). Buffer was exchanged with 0.1 M Tris pH 7.5, 1 mM NaCl, 5 mM DTT during concentration by ultrafiltration. Protein concentration was determined using Advanced Protein Assay Reagent (Cytoskeleton, Denver CO) with γ-globulins (Sigma-Aldrich) as the standard. Purified protein was visualized *via* Coomassie stained SDS-PAGE. The yield of *MtbDXR* and *PfDXR* averaged 5 mg and 1 mg per 1 L shake flask, respectively.

DXR enzyme inhibition assay

P. falciparum DXR activity was assayed at 37 °C by spectrophotometrically monitoring the enzyme catalyzed oxidation of NADPH upon addition of 1-deoxy-D-xylulose 5-phosphate (DOXP; Echelon Biosciences, Salt Lake City, UT) to the assay mixture, as described previously.^{47,48} Briefly, the assay system contained 100 mM Tris pH 7.8, 25 mM MgCl₂, 0.86 μM *PfDXR*, and 150 μM NADPH. The reaction was initiated by adding 144 μM DOXP to the complete assay mixture. For the *MtbDXR* assays, the assay contained 100 mM MES pH 6.5, 25 mM MgCl₂, 0.89 μM *MtbDXR*, 150 μM NADPH, and 47 μM DOXP. The oxidation of NADPH was monitored at 340 nm using an Agilent 8453 UV-visible spectrophotometer equipped with a temperature regulated cuvette holder. All assays were performed in technical duplicates. All determined IC₅₀ values were independently validated (by a second enzymologist) to be within 5% of the determined value. If they were not, then additional replicate assays were performed to further refine the dose-response plots (and resulting IC₅₀ value) until validation within the 5% cutoff was achieved.

P. falciparum growth inhibition assay

Asynchronous *P. falciparum* cultures were maintained in 2% hematocrit suspended in complete media (RPMI-1640 (Millipore-Sigma) supplemented with 27 mM sodium bicarbonate, 11 mM glucose, 5 mM HEPES, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 0.01 mM thymidine, 10 μg mL^{−1} gentamycin, and 0.5% Albumax (Life Technologies)) under 5% O₂/5% CO₂/90% N₂ atmosphere at 37 °C. Wild type strain 3D7 (BEI Resources, MRA-102) and fosmidomycin resistant strain (*had1*) E1-C12 (ref. 58) were diluted to 0.5% parasitemia and treated with inhibitors at concentrations ranging from 1.2 nM to 492.4 μM at the start of assays. Growth inhibition assays were performed in opaque 96-well



plates at 100 μL culture volume. After 3 days, parasite growth was quantified by measuring DNA content using PicoGreen (Life Technologies) as described.⁵⁸ Fluorescence was measured on a FLUOstar Omega microplate reader (BMG Labtech) at 485 nm excitation and 528 nm emission. Half-maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression analysis using GraphPad Prism software. Data was fit using a 4-parameter dose-response curve. For isopentenyl pyrophosphate (IPP) (Isoprenoids, LC) rescue experiments, 125 μM IPP was added to the appropriate wells for the duration of the experiment. Each assay was performed as a technical duplicate. Each compound was assayed as a biological triplicate.

M. tuberculosis growth inhibition assay

A stock culture of *Mtb* H37Rv (ATCC 27294) was grown to OD 0.5 in 7H9/glucose/casitone/tyloxapol consisting of Middlebrook 7H9 broth (Difco) supplemented with 0.08% NaCl, 0.4% glucose, 0.05% Bacto casitone and 0.05% tyloxapol. The culture was diluted 1:500 in 7H9/glucose/casitone/tyloxapol before aliquoting 50 μL into each well of a 96-well plate. The inhibitors were dissolved in DMSO to make stock solutions of 20 mg mL^{-1} . Inhibitors were added to the first row of wells of the 96-well plate with 100 μL of 7H9/glucose/casitone/tyloxapol with remaining wells containing 50 μL of 7H9/glucose/casitone/tyloxapol. After pipet mixing and use of a multichannel pipet, 50 μL was removed from each well in the first row and added to the second row. 2-Fold dilution in this manner was carried out to give eleven dilutions of each inhibitor. The plates were incubated for 2 weeks at 37 $^{\circ}\text{C}$, and the MIC_{99} values were read macroscopically using an inverted plate reader. Each measurement was made three independent times.

HepG2 cell inhibition assay

For cytotoxicity assays, HepG2 cells (ATCC HB-8065) were grown in DMEM supplemented with 4 mM L-glutamine (Gibco #11966-025) with 4.5 g L^{-1} D-glucose as carbon source. Cells were trypsinized, resuspended in DMEM/glutamine/glucose to 4×10^5 cells per mL and 50 μL per well transferred to flat-bottom white opaque tissue culture plates (Falcon #353296) containing 50 μL per well of the respective medium with test compound. Compound concentrations were two-fold dilutions ranging from 100 to 0.1 $\mu\text{g mL}^{-1}$ as well as the drug-free DMSO-only control. All concentrations were tested in duplicate. After 24 h incubation at 5% CO_2 , 37 $^{\circ}\text{C}$, 10 μL per well of Celltiter-Glo reagent (Promega #G9241) was added and luminescence recorded after 20 min incubation in the dark.

Conflicts of interest

The authors declare no competing financial interest.

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References

- 1 *World Malaria Report 2022*, World Health Organization, Geneva, 2022, <https://www.who.int/publications/i/item/9789240064898>.
- 2 *Global Tuberculosis Report 2022*, World Health Organization, Geneva, 2022, <https://www.who.int/teams/global-tuberculosis-programme/tb-reports>.
- 3 A. Sharma, M. De Rosa, N. Singla, G. Singh, R. Barnwal and A. Pandey, Tuberculosis: An Overview of the Immunogenic Response, Disease Progression, and Medicinal Chemistry Efforts in the Last Decade toward the Development of Potential Drugs for Extensively Drug-Resistant Tuberculosis Strains, *J. Med. Chem.*, 2021, **64**, 4359–4395.
- 4 S. Wellington and D. Hung, The Expanding Diversity of Mycobacterium tuberculosis Drug Targets, *ACS Infect. Dis.*, 2018, **4**, 696–714.
- 5 G. Pines, E. Oh, M. Bassalso, A. Choudhury, A. Garst, R. Fankhauser, C. Eckert and R. Gill, Genomic Deoxyxylulose Phosphate Reductoisomerase (DXR) Mutations Conferring Resistance to the Antimalarial Drug Fosmidomycin in *E. coli*, *ACS Synth. Biol.*, 2018, **7**, 2824–2832.
- 6 B. Rigel and P. Roepe, Altered Drug Transport by *Plasmodium falciparum* Chloroquine Resistance Transporter Isoforms Harboring Mutations Associated with Piperaquine Resistance, *Biochemistry*, 2020, **59**, 2484–2493.
- 7 J. Okombo and K. Chibale, Insights into Integrated Lead Generation and Target Identification in Malaria and Tuberculosis Drug Discovery, *Acc. Chem. Res.*, 2017, **50**, 1606–1616.
- 8 J. McKie, K. Douglas, C. Chan, S. Roser, R. Yates, M. Read, J. Hyde, M. Dascombe, Y. Yuthavong and W. Sirawaporn, Rational Drug Design Approach for Overcoming Drug Resistance: Application to Pyrimethamine Resistance in Malaria, *J. Med. Chem.*, 1998, **41**, 1367–1370.
- 9 V. Singh and K. Chibale, Strategies to Combat Multi-Drug Resistance in Tuberculosis, *Acc. Chem. Res.*, 2021, **54**, 2361–2376.
- 10 G. Pines, E. J. Oh, M. C. Bassalo, A. Choudhury, A. D. Garst, R. G. Fankhauser, C. A. Eckert and R. T. Gill, Genomic Deoxyxylulose Phosphate Reductoisomerase (DXR) Mutations Conferring Resistance to the Antimalarial Drug Fosmidomycin in *E. Coli*, *ACS Synth. Biol.*, 2018, **7**, 2824–2832.
- 11 T. Kuzuyama, T. Shimizu, S. Takahashi and H. Seto, Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate



- pathway for terpenoid biosynthesis, *Tetrahedron Lett.*, 1998, **39**, 7913–7916.
- 12 L. Kuntz, D. Tritsch, C. Grosdemange-Billiard, A. Hemmerlin, A. Willem, T. J. Bach and M. Rohmer, Isoprenoid biosynthesis as a target for antibacterial and antiparasitic drugs: phosphonohydroxamic acids as inhibitors of deoxyxylulose phosphate reductoisomerase, *Biochem. J.*, 2005, **386**, 127–135.
 - 13 S. Takahashi, T. Kuzuyama, H. Watanabe and H. Seto, A 1-deoxy-d-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-d-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 9879–9884.
 - 14 X. Wang and C. S. Dowd, The Methylerythritol Phosphate Pathway: Promising Drug Targets in the Fight against Tuberculosis, *ACS Infect. Dis.*, 2018, **4**, 278–290.
 - 15 A. Frank and M. Groll, The Methylerythritol Phosphate Pathway to Isoprenoids, *Chem. Rev.*, 2017, **117**, 5675–5703.
 - 16 A. Argyrou and J. S. Blanchard, Kinetic and Chemical Mechanism of Mycobacterium Tuberculosis 1-Deoxy-D-Xylulose-5-Phosphate Isomeroreductase, *Biochemistry*, 2004, **43**, 4375–4384.
 - 17 T. Kuzuyama, T. Shimizu, S. Takahashi and H. Seto, Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis, *Tetrahedron Lett.*, 1998, **39**, 7913–7916.
 - 18 E. R. Jackson, G. San Jose, R. C. Brothers, E. K. Edelstein, Z. Sheldon, A. Haymond, C. Johny, H. I. Boshoff, R. D. Couch and C. S. Dowd, The effect of chain length and unsaturation on *Mtb* Dxr inhibition and antitubercular killing activity of FR900098 analogs, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 649–653.
 - 19 R. L. Edwards, R. C. Brothers, X. Wang, M. I. Maron, P. D. Ziniel, P. S. Tsang, T. E. Kraft, P. W. Hruz, K. C. Williamson, C. S. Dowd and A. R. O. John, MEPicides: Potent Antimalarial Prodrugs Targeting Isoprenoid Biosynthesis, *Sci. Rep.*, 2017, **7**, 8400.
 - 20 T. Masini and A. K. H. Hirsch, Development of Inhibitors of the 2 C-Methyl-D-Erythritol 4-Phosphate (MEP) Pathway Enzymes as Potential Anti-Infective Agents, *J. Med. Chem.*, 2014, **57**, 9740–9763.
 - 21 N. Singh, G. Cheve, M. Avery and C. McCurdy, Comparative Protein Modeling of 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase Enzyme from Plasmodium falciparum: A Potential Target for Antimalarial Drug Discovery, *J. Chem. Inf. Model.*, 2006, **46**, 1360–1370.
 - 22 J. Munos, X. Pu, S. Mansoorabadi, H. Kim and H. Liu, A Secondary Kinetic Isotope Effect Study of the 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase-Catalyzed, *J. Am. Chem. Soc.*, 2009, **131**, 2048–2049.
 - 23 L. Deng, K. Endo, M. Kato, G. Cheng, S. Yajima and Y. Song, Structures of 1-Deoxy-D-Xylulose5-Phosphate Reductoisomerase/Lipophilic Phosphonate Complexes, *ACS Med. Chem. Lett.*, 2011, **2**, 165–170.
 - 24 A. Wong, J. W. Munos, V. Devasthali, K. A. Johnson and H. Liu, Study of 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase: Synthesis and Evaluation of Fluorinated Substrate Analogues, *Org. Lett.*, 2004, **6**, 3625–3628.
 - 25 B. Lell, R. Ruangweerayut, J. Wiesner, M. A. Missinou, A. Schindler, T. Baranek, M. Hintz, D. Hutchinson, H. Jomaa and P. G. Kremsner, Fosmidomycin, a novel chemotherapeutic agent for malaria, *Antimicrob. Agents Chemother.*, 2003, **47**, 735–738.
 - 26 T. Umeda, N. Tanaka, Y. Kusakabe, M. Nakanishi, Y. Kitade and K. T. Nakamura, Molecular basis of fosmidomycin's action on the human malaria parasite *Plasmodium falciparum*, *Sci. Rep.*, 2011, **1**, 1–8.
 - 27 A. M. Jansson, A. Wieckowska, C. Bjorkelid, S. Yahiaoui, S. Sooriyaarachchi, M. Lindh and S. L. Mowbray, DXR inhibition by potent mono- and disubstituted fosmidomycin analogues, *J. Med. Chem.*, 2013, **56**, 6190–6199.
 - 28 B. Zhang, K. Watts, D. Hodge, L. Kemp, D. Hunstad, L. Hicks and A. Odom, A Second Target of the Antimalarial and Antibacterial Agent Fosmidomycin Revealed by Cellular Metabolic Profiling, *Biochemistry*, 2011, **50**, 3570–3577.
 - 29 E. Uh, E. Jackson, G. San Jose, M. Maddox, R. Lee, R. Lee, H. Boshoff and C. Dowd, Antibacterial and antitubercular activity of fosmidomycin, FR900098, and their lipophilic analogs, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 6973–6976.
 - 30 S. Kholodar, G. Tomblin, J. Liu, Z. Tan, C. Allen, A. Gulick and A. Murkin, Alteration of the Flexible Loop in 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase Boosts Enthalpy-Driven Inhibition by Fosmidomycin, *Biochemistry*, 2014, **53**, 3423–3431.
 - 31 A. Leon, L. Liu, Y. Yang, M. P. Hudock, P. Hall, F. Yin, D. Studer, K.-J. Puan, C. T. Morita and E. Oldfield, Isoprenoid Biosynthesis as a Drug Target: Bisphosphonate Inhibition of Escherichia Coli K12 Growth and Synergistic Effects of Fosmidomycin, *J. Med. Chem.*, 2006, **49**, 7331–7341.
 - 32 C. Zinglé, L. Kuntz, D. Tritsch, C. Grosdemange-Billiard and M. Rohmer, Isoprenoid Biosynthesis via the Methylerythritol Phosphate Pathway: Structural Variations around Phosphonate Anchor and Spacer of Fosmidomycin, a Potent Inhibitor of Deoxyxylulose Phosphate Reductoisomerase, *J. Org. Chem.*, 2010, **75**, 3203–3207.
 - 33 M. Andaloussi, L. M. Henriksson, A. Wieckowska, M. Lindh, C. Bjorkelid, A. M. Larsson, S. Suresh, H. Iyer, B. R. Srinivasa, T. Bergfors, T. Unge, S. L. Mowbray, M. Larhed, T. A. Jones and A. Karlen, Design, synthesis, and X-ray crystallographic studies of alpha-aryl substituted fosmidomycin analogues as inhibitors of Mycobacterium tuberculosis 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *J. Med. Chem.*, 2011, **54**, 4964–4976.
 - 34 G. San Jose, E. R. Jackson, A. Haymond, C. Johny, R. L. Edwards, X. Wang, R. C. Brothers, E. K. Edelstein, A. R. Odom, H. I. Boshoff, R. D. Couch and C. S. Dowd, Structure-Activity Relationships of the MEPicides: N-Acyl and O-Linked Analogs of FR900098 as Inhibitors of Dxr from Mycobacterium tuberculosis and Yersinia pestis, *ACS Infect. Dis.*, 2016, **2**, 923–935.



- 35 H. S. Ball, M. Girma, M. Zainab, H. Riley, C. T. Behrendt, C. Lienau, S. Konzuch, L. A. A. Avelar, B. Lungerich, I. Soojhawon, S. M. Noble, T. Kurz and R. D. Couch, Inhibition of the *Yersinia Pestis* Methylerythritol Phosphate Pathway of Isoprenoid Biosynthesis by α -Phenyl-Substituted Reverse Fosmidomycin Analogues, *ACS Omega*, 2020, 5, 5170–5175.
- 36 T. Verbruggen, P. Vandum, J. Pouyez, L. Maes, J. Wouters and S. Van Calenbergh, Alpha-Heteroatom Derivatized Analogues of 3-(Acetylhydroxyamino)propyl Phosphonic Acid (FR900098) as Antimalarials, *J. Med. Chem.*, 2013, 56, 376–380.
- 37 X. Wang, R. L. Edwards, H. Ball, C. Johnson, A. Haymond, M. Girma, M. Manikkam, R. C. Brothers, K. T. McKay, S. D. Arnett, D. M. Osbourn, S. Alvarez, H. I. Boshoff, M. J. Meyers, R. D. Couch, A. R. Odom John and C. S. Dowd, MEPicides: α,β -Unsaturated Fosmidomycin Analogues as DXR Inhibitors against Malaria, *J. Med. Chem.*, 2018, 61, 8847–8858.
- 38 M. B. Girma, H. S. Ball, X. Wang, R. C. Brothers, E. R. Jackson, M. J. Meyers, C. S. Dowd and R. D. Couch, Mechanism of Action of N-Acyl and N-Alkoxy Fosmidomycin Analogs: Mono- and Bisubstrate Inhibition of IspC from *Plasmodium Falciparum*, a Causative Agent of Malaria, *ACS Omega*, 2021, 6, 27630–27639.
- 39 S. Konzuch, T. Umeda, J. Held, S. Hähn, K. Brücher, C. Lienau, C. T. Behrendt, T. Gräwert, A. Bacher, B. Illarionov, M. Fischer, B. Mordmüller, N. Tanaka and T. Kurz, Binding Modes of Reverse Fosmidomycin Analogs toward the Antimalarial Target IspC, *J. Med. Chem.*, 2014, 57, 8827–8838.
- 40 L. Deng, S. Sundriyal, V. Rubio, Z. Shi and Y. Song, Coordination Chemistry Based Approach to Lipophilic Inhibitors of 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase, *J. Med. Chem.*, 2009, 52, 6539–6542.
- 41 S. Yajima, K. Hara, J. M. Sanders, F. Yin, K. Ohsawa, J. Wiesner, H. Jomaa and E. Oldfield, Crystallographic Structures of Two Bisphosphonate:1-Deoxyxylulose-5-Phosphate Reductoisomerase Complexes, *J. Am. Chem. Soc.*, 2004, 126, 10824–10825.
- 42 A. Leon, L. Liu, Y. Yang, M. P. Hudock, P. Hall, F. Yin, D. Studer, K.-J. Puan, C. T. Morita and E. Oldfield, Isoprenoid Biosynthesis as a Drug Target: Bisphosphonate Inhibition of *Escherichia Coli* K12 Growth and Synergistic Effects of Fosmidomycin, *J. Med. Chem.*, 2006, 49, 7331–7341.
- 43 C. Zinglé, L. Kuntz, D. Tritsch, C. Grosdemange-Billiard and M. Rohmer, Isoprenoid Biosynthesis via the Methylerythritol Phosphate Pathway: Structural Variations around Phosphonate Anchor and Spacer of Fosmidomycin, a Potent Inhibitor of Deoxyxylulose Phosphate Reductoisomerase, *J. Org. Chem.*, 2010, 75, 3203–3207.
- 44 T. A. Wenciewicz, B. Yang, J. R. Rudloff, A. G. Oliver and M. J. Miller, N–O Chemistry for Antibiotics: Discovery of N-Alkyl-N-(Pyridin-2-Yl)Hydroxylamine Scaffolds as Selective Antibacterial Agents Using Nitroso Diels–Alder and Ene Chemistry, *J. Med. Chem.*, 2011, 54, 6843–6858.
- 45 T. Knak, M. A. Abdullaziz, S. Höfmann, L. A. Alves Avelar, S. Klein, M. Martin, M. Fischer, N. Tanaka and T. Kurz, Over 40 Years of Fosmidomycin Drug Research: A Comprehensive Review and Future Opportunities, *Pharmaceuticals*, 2022, 15, 1553.
- 46 K. Brücher, T. Gräwert, S. Konzuch, J. Held, C. Lienau, C. Behrendt, B. Illarionov, L. Maes, A. Bacher, S. Wittlin, B. Mordmüller, M. Fischer and T. Kurz, Prodrugs of Reverse Fosmidomycin Analogues, *J. Med. Chem.*, 2015, 58, 2025–2035.
- 47 C. T. Behrendt, A. Kunfermann, V. Illarionova, A. Matheessen, M. K. Pein, T. Gräwert, J. Kaiser, A. Bacher, W. Eisenreich, B. Illarionov, M. Fischer, L. Maes, M. Groll and T. Kurz, Reverse Fosmidomycin Derivatives against the Antimalarial Drug Target IspC (Dxr), *J. Med. Chem.*, 2011, 54, 6796–6802.
- 48 L. Deng, K. Endo, M. Kato, G. Cheng, S. Yajima and Y. Song, Structures of 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase/Lipophilic Phosphonate Complexes, *ACS Med. Chem. Lett.*, 2011, 2, 165–170.
- 49 A. Wong, J. W. Munos, V. Devasthali, K. A. Johnson and H. Liu, Study of 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase: Synthesis and Evaluation of Fluorinated Substrate Analogues, *Org. Lett.*, 2004, 6, 3625–3628.
- 50 R. Chofor, S. Sooriyaarachchi, M. D. P. Risseuw, T. Bergfors, J. Pouyez, C. Johny, A. Haymond, A. Everaert, C. S. Dowd, L. Maes, T. Coenye, A. Alex, R. D. Couch, T. A. Jones, J. Wouters, S. L. Mowbray and S. Van Calenbergh, Synthesis and Bioactivity of β -Substituted Fosmidomycin Analogues Targeting 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase, *J. Med. Chem.*, 2015, 58, 2988–3001.
- 51 A. Nordqvist, C. Björkelid, M. Andaloussi, A. M. Jansson, S. L. Mowbray, A. Karlén and M. Larhed, Synthesis of Functionalized Cinnamaldehyde Derivatives by an Oxidative Heck Reaction and Their Use as Starting Materials for Preparation of *Mycobacterium Tuberculosis* 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase Inhibitors, *J. Org. Chem.*, 2011, 76, 8986–8998.
- 52 G. Cai, L. Deng, B. G. Fryszczyn, N. G. Brown, Z. Liu, H. Jiang, T. Palzkill and Y. Song, Thermodynamic Investigation of Inhibitor Binding to 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase, *ACS Med. Chem. Lett.*, 2012, 3, 496–500.
- 53 K. Brücher, B. Illarionov, J. Held, S. Tschan, A. Kunfermann, M. K. Pein, A. Bacher, T. Gräwert, L. Maes, B. Mordmüller, M. Fischer and T. Kurz, α -Substituted β -Oxa Isosteres of Fosmidomycin: Synthesis and Biological Evaluation, *J. Med. Chem.*, 2012, 55, 6566–6575.
- 54 G. San Jose, E. R. Jackson, E. Uh, C. Johny, A. Haymond, L. Lundberg, C. Pinkham, K. Kehn-Hall, H. I. Boshoff, R. D. Couch and C. S. Dowd, Design of Potential Bisubstrate Inhibitors against *Mycobacterium tuberculosis* (Mtb) 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase (Dxr)-Evidence of a Novel Binding Mode, *MedChemComm*, 2013, 4, 1099–1104.
- 55 T. Haemers, J. Wiesner, S. Van Poecke, J. Goeman, D. Henschker, E. Beck, H. Jomaa and S. Van Calenbergh, Synthesis of α -substituted fosmidomycin analogues as highly



- potent *Plasmodium falciparum* growth inhibitors, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 1888–1891.
- 56 T. Haemers, J. Wiesner, R. Busson, H. Jomaa and S. Van Calenbergh, Synthesis of α -Aryl-Substituted and Conformationally Restricted Fosmidomycin Analogues as Promising Antimalarials, *Eur. J. Org. Chem.*, 2006, **2006**, 3856–3863.
 - 57 E. Yeh and J. DeRisi, Chemical Rescue of Malaria Parasites Lacking an Apicoplast Defines Organelle Function in Blood-Stage *Plasmodium falciparum*, *PLoS Biol.*, 2011, **9**, e1001138.
 - 58 A. M. Guggisberg, J. Park, R. L. Edwards, M. L. Kelly, D. M. Hodge, N. H. Tolia and A. R. Odom, A sugar phosphatase regulates the methylerythritol phosphate (MEP) pathway in malaria parasites, *Nat. Commun.*, 2014, **5**, 4467.
 - 59 X. Wang, R. L. Edwards, H. S. Ball, K. M. Heidel, R. C. Brothers, C. Johnson, A. Haymond, M. Girma, A. Dailey, J. S. Roma, H. I. Boshoff, D. M. Osbourn, M. J. Meyers, R. D. Couch, A. R. Odom John and C. S. Dowd, MEPicides: α,β Unsaturated Fosmidomycin N-Acyl Analogs as Efficient Inhibitors of *Plasmodium Falciparum* 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase, *ACS Infect. Dis.*, 2023, **9**(7), 1387–1395.
 - 60 A. Haymond, C. Johny, T. Dowdy, B. Schweibenz, K. Villarroel, R. Young, C. J. Mantooth, T. Patel, J. Bases, G. S. Jose, E. R. Jackson, C. S. Dowd and R. D. Couch, Kinetic Characterization and Allosteric Inhibition of the *Yersinia Pestis* 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase (MEP Synthase), *PLoS One*, 2014, **9**(8), e106243.

