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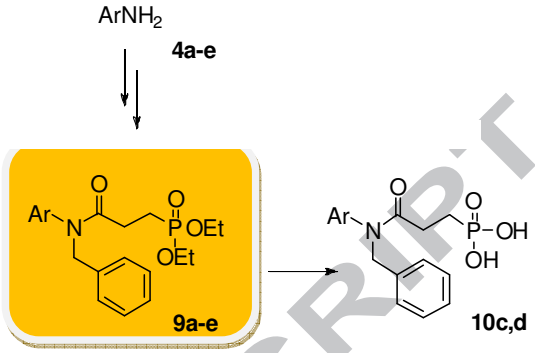


GRAHICAL ABSTRACT

Synthesis and Antimalarial Activity of *N*-Benzylated (*N*-Arylcarbamoyl)alkylphosphonic Acid Derivatives

Christiana M. Adeyemi,[a] Faridoon,[a] Michelle Isaacs,[c] Dumisani Mnkandhla,[c] Heinrich C. Hoppe,[b,c] Rui W.M. Krause[a,c] and Perry T. Kaye[a,c]*

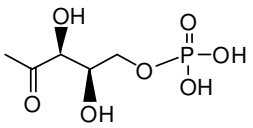
[a] Department of Chemistry, [b] Department of Biochemistry and Microbiolgy and [c] Centre for Chemico- and Biomedicinal Research, Rhodes University, Grahamstown, 6140, South Africa.

Abstract. A series of novel and readily accessible *N*-benzylated (*N*-arylcarbamoyl)-alkylphosphonate esters and related compounds have been prepared as potential antimalarial agents. Bioassays reveal that some of these compounds exhibit promising activity against *Plasmodium falciparum*, and exhibit no significant growth inhibition of HeLa cells.

Keywords: Anti-malarial, *N*-benzylated phosphonate esters, *Pf*DXR, aryl and heteroaryl derivatives

1. Introduction

The scourge of malaria continues to be a major health challenge in many countries, and there are concerns that global warming might extend the habitat of infectious mosquito vectors into regions which are, at present, malaria-free.[1,2] The development of resistance to established antimalarial drugs has exacerbated the problem and necessitates the identification of new drug targets and the development of new and effective drugs.[3] 1-Deoxy-1-D-xylulose-5-phosphate (DOXP; **1**) reductoisomerase (*Pf*DXR),[4,5] is a critical enzyme in the non-mevalonate, isoprenoid biosynthetic pathway in *Plasmodium falciparum* (*Pf*), it is parasite-specific and there is considerable interest in developing effective inhibitors of this enzyme as potential anti-malarial drugs.



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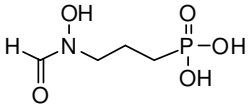

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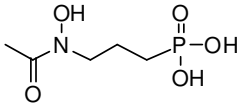

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Corresponding author: Prof Perry Kaye. E-mail: P.Kaye@ru.ac.za

In our own research in this area, we have been exploring the synthesis and DXR-inhibition potential of simplified and readily accessible analogues of fosmidomycin **2** – a naturally occurring DXR inhibitor and its acetyl derivative FR900098 **3**.[6,7] Results from studies involving *N*-aryl- and *N*-heteroarylcarboxamides have supported the importance of no more than two methylene groups in the 'hydrophobic patch' between the metal chelating and phosphonate moieties, and have indicated the presence of additional hydrophobic pockets adjacent to the *Pf*DXR active site.[7] Kurz and co-workers [8] have reported the DXR inhibition potential of 'reverse' fosmidomycin analogues in which the carbonyl and hydroxylamine moieties are transposed. In this communication, we now report the preparation and evaluation of a series of *N*-benzylated 'reverse' fosmidomycin analogues, including *N*-benzylated derivatives of compounds from our earlier studies[9] which gave the best, albeit very modest, *Ec*DXR- and *Pf*DXR-inhibition data. The key structural features of these analogues, compared to fosmidomycin **2**, are summarised in Figure 1 and include:- i) a common phosphonate moiety as a replacement for the more labile phosphate group present in the natural DXR substrate DOXP **1**; ii) a bimethylene hydrophobic patch rather than the trimethylene spacer in fosmidomycin **2**; iii) an amide group as a reverse hydroxamate replacement; and iv) a hydrophobic, *N*-benzyl group to occupy a hydrophobic pocket adjacent to the DXR active site.

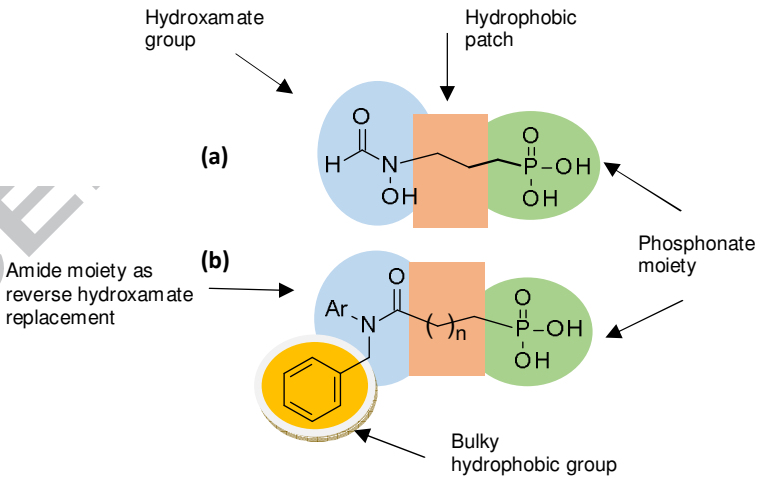
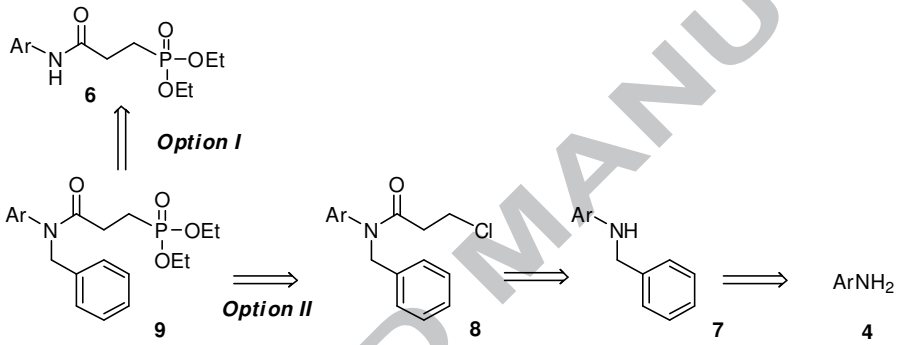


Figure 1. Structural similarities and differences between: (a) fosmidomycin **2**; and (b) scaffolds used in the present study.

2. Results and Discussion

2.1. Chemistry

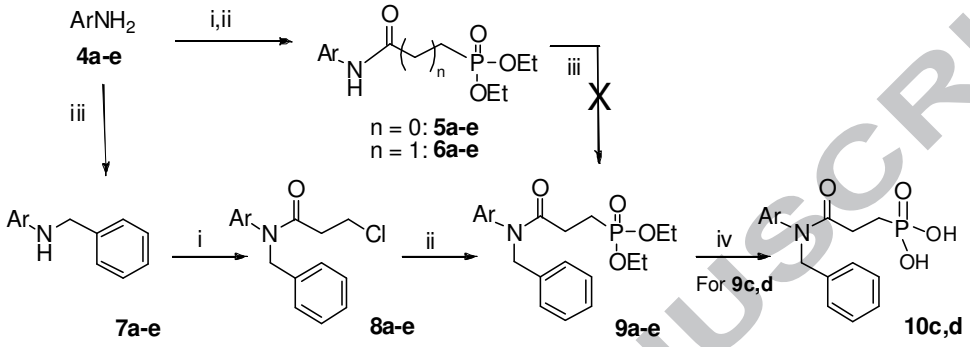
It was envisaged that the desired *N*-benzylated, bi-methylene-bridged phosphonate esters **9** (or their mono-methylene-bridged analogues) could be accessed by direct benzylation (*retrosynthetic option I*, Scheme 1) of the amide nitrogen in the phosphonate esters **6** (or their mono-methylene-bridged analogues **5**), which had been synthesised previously (Scheme 2).[6,7] However, reactions of selected phosphonate esters with benzyl bromide in the presence of base failed to afford the *N*-benzylated derivatives,[10] and attention was turned to *retrosynthetic option II* (Scheme 1). In this approach, *N*-benzylation is effected first.



Scheme 1. Retrosynthetic options to access the target molecules **9**.

Thus, mixtures of each of the primary amines **4a-e**, benzyl bromide (1 eq.) and $NaHCO_3$ (1 eq.) in dry THF (Scheme 2) were stirred overnight at room temperature under an inert atmosphere. Work-up and flash chromatography afforded the *N*-benzylated derivatives **7a-e** in yields of up to 72% (Table 1). In addition to the expected product **7b**, the *N*,*O*-bis-benzylated analogue, *N*-benzyl-3-[(benzyloxy)methyl]aniline **11** was also obtained as a new but minor product (19%) from the reaction with 3-(hydroxymethyl)aniline **4b**. Acylation of the *N*-benzylated amines **7a-e** was effected in THF using NaH, as base, and 3-chloropropanoyl chloride. Work-up and flash-chromatography afforded the corresponding, novel 3-chloropanamides **8a-e** in low to moderate yield; the bis-acylated analogue **12** of the phenolic derivative **8d** was also obtained as a minor product (14%). Michaelis-Arbuzov reactions of the 3-chloropanamides **8a-e** in refluxing triethyl phosphite led to the series of novel phosphonate esters **9a-e** which were obtained as yellow oils in yields of up to 59%

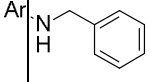
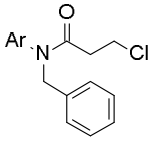
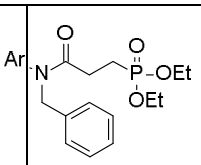
(Table 1) following flash- or preparative layer chromatography. The common method of removing excess triethyl phosphite by washing with hexane was precluded by the solubility of the products in hexane and, consequently, the crude products were purified chromatographically. Given the design criteria discussed in the Introduction, attention was focussed on the preparation of the *bi-methylene-bridged*, *N*-benzylated phosphonate esters **9.**



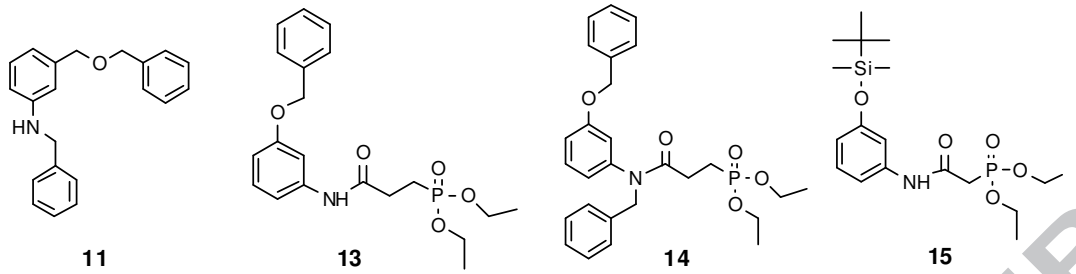
Scheme 2.

Reagents and conditions:- (i) NaH, dry THF, then 3-chloropropanoyl chloride, r.t.; (ii) triethyl phosphite, 6 h, 120-150 ºC; (iii) $NaHCO_3$, BnBr, dry THF; (iv) TMSBr, dry DCM, N_2, 24 h.

Table 1. Percentage yields obtained for the *N*-benzylated derivatives **7a-e**, the 3-chloropopanamides **8a-e** and the phosphonate esters **9a-e**.

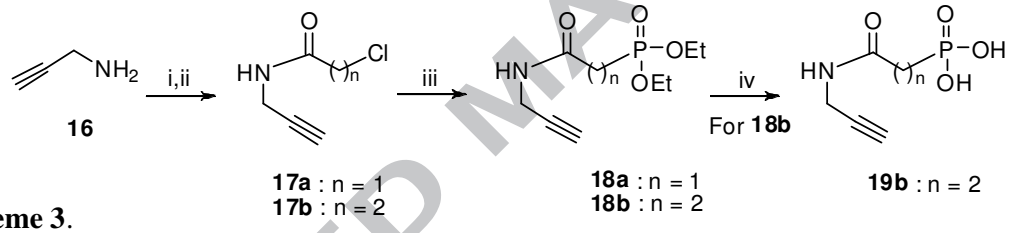
Ar			
3-Bromophenyl	**7a** 65	**8a** 50	**9a** 51
3-(Hydroxymethyl)phenyl	**7b** 63 [a]	**8b** 36	**9b** 47
2-Pyridyl	**7c** 56	**8c** 33	**9c** 49
3-Hydroxyphenyl	**7d** 62	**8d** 39 [b]	**9d** 51
3-Nitrophenyl	**7e** 72	**8e** 52	**9e** 59

[a] Bis-benzylated analogue **11** (19%). [b] Bis-2-chloropropanoyl analogue **12** (14%).



11 **13** **14** **15**

Additional compounds obtained in this study and evaluated for anti-malarial activity include:- i) the phosphonate esters **13** and **14** obtained by benzylation of compound **6d** (Scheme 2); ii) the *O*-TBDMS-protected methylphosphonate ester **15**; and iii) the phosphonated *N*-(2-propynyl)carboxamides **18a,b**, prepared from propargylamine **16** (Scheme 3).



16 **17a** : n = 1, **17b** : n = 2 **18a** : n = 1, **18b** : n = 2 **19b** : n = 2

Scheme 3.

Reagents and conditions:- (i) triethylamine, dry THF, 0 ^{o}C; (ii) chloroacetyl chloride or 3-chloropropanoyl chloride; (iii) triethyl phosphite 6 h, 120-150 ºC; and iv) TMSBr, dry DCM, N_2, 24 h.

While the purified phosphonic esters could be used directly as possible pro-drugs in the *P.falciparum* parasite (pLDH) bioassays, hydrolysis to the corresponding acids was required for the *in vitro* evaluation of *Pf*-DXR inhibition potential – the *Pf*-DXR enzyme lacking the necessary esterases. Hydrolysis of three of the most active esters (**9c**, **9d and 18b**) was effected, using five equivalents of bromotrimethylsilane in dry DCM under nitrogen,[8] and afforded the corresponding phosphonic acids **10c**, **10d** and **19b**.

2.2. *Pf* parasite and *Pf*-DXR Inhibition and Toxicity Assays

Cytotoxicity (using HeLa cells) and malaria parasite lactate dehydrogenase (pLDH) assays were first conducted using 20 µM solutions of the synthesised compounds in triplicate, and the results are illustrated in Figure 2 and summarised in Table 2. The phosphonate esters appear to exhibit minimal growth inhibition of the HeLa cells and, in most cases, 100% HeLa cell viability was observed. IC_{50} values were then determined for compounds **5b**, **5c**, **18b**, **14**, **9c** and **9d** (all of which inhibited the parasite completely at a ligand concentration of 20 µM) and for compound **14** (which, at 20 µM, exhibited < 20% malaria parasite viability). The percentage parasite cell viability was plotted against the logarithm of the ligand concentration (Figure 3) and IC_{50} values were determined using the trend-line. Surprisingly, the non-benzylated compound **5b**, which contains only one methylene group in the hydrophobic patch, has the lowest IC_{50} value at 10.1 µM. Under the same conditions, chloroquine exhibited an IC_{50} value of 0.07 µM. The remaining ligands also gave encouraging IC_{50} values in the low µM range (**5c**: 23.1; **9c**: 22.3; **9d**: 15.9; **14**: 16.4; **18b**: 20.1).

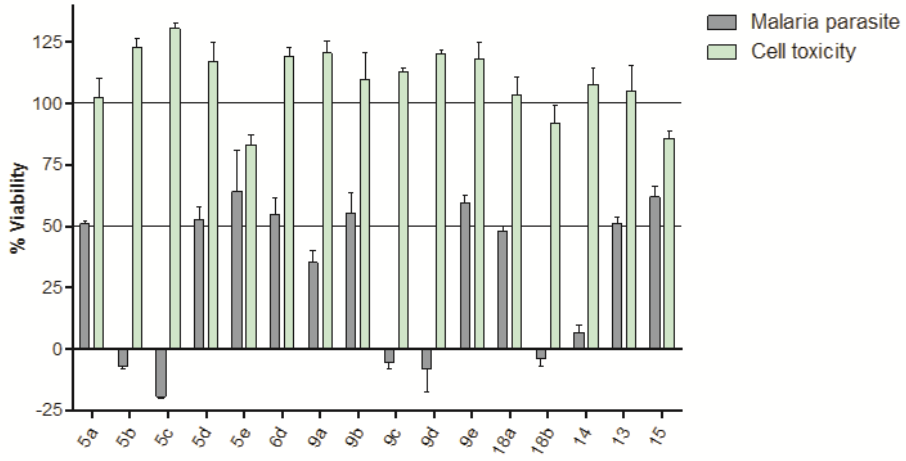


Figure 2. Cell toxicity (HeLa cell viability) screening and anti-malarial (*P.falciparum* pLDH) assay of the synthesised phosphonate esters (20 µM).

Table 2. Residual % *P.falciparum* viability and toxicity (HeLa cell viability) data for 20 µM solutions of the synthetic ligands.

Compound	*Plasmodium falciparum* Viability %	SD	HeLa Viability %	SD
5a	50.91	0.96	102.44	7.81
5b	-7.26	0.80	122.76	3.92
5c	-19.39	0.64	130.59	2.06
5d	52.42	5.67	116.80	7.91
5e	64.13	16.90	83.14	4.07
6d	54.54	6.98	119.28	3.72
9a	35.19	4.95	120.60	4.85
9b	55.44	8.06	109.67	10.90
9c	-5.33	2.56	112.93	1.47
9d	-8.05	9.66	120.15	1.81
9e	59.60	2.96	118.23	6.46
13	51.13	2.37	104.83	10.50
14	6.84	2.87	107.74	6.45
15	61.87	4.42	85.68	3.01
18a	47.96	1.92	103.65	6.98
18b	-4.04	3.02	91.95	7.00

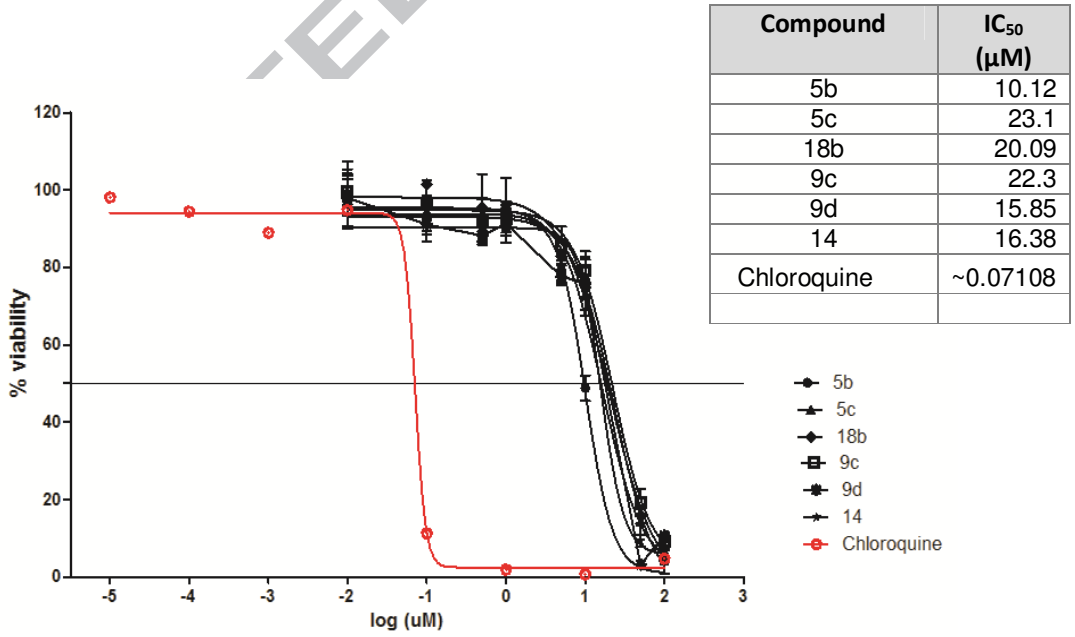


Figure 3. The inhibitory effect of selected compounds, showing percentage viability of the *P.falciparum* parasite at various concentrations and corresponding IC_{50} values.

The bioassay results reveal a number of interesting features.

i) Six of the phosphonate esters (**5b**, **5c**, **9c, 9d**, **14** and **18b**) exhibit encouraging inhibition of the parasite and very low toxicity at a concentration of 20 μM. In fact, five of these ligands appear to exhibit *ca*. 0% *Pf* viability at this concentration!

ii) Further detailed analysis of the six most active ligands reveals their IC_{50} values to be in the low μmolar range (10 - 23).

iii) Unexpectedly, introduction of the *N*-benzyl group coupled with the presence of a bi-methylene bridge, aimed at enhancing *Pf*DXR inhibition, appears to have increased inhibitory activity against the *Pf* parasite in only one case; the *N*-benzylated ligand **9d** exhibits significantly better inhibitory activity (*ca*. 0% *Pf* viability at 20 μM) than the non-benzylated, mono-methylene-bridged analogue **5d** (52% *Pf* viability at 20 μM).

iv) Increasing the length of the hydrophobic patch between the amide and phosphonate ester moieties (from one to two methylene groups) in the phosphonated *N*-(2-propynyl)carboxamides clearly enhances inhibition activity significantly (*Pf* viability: **18a**: 48%; **18b**: *ca*. 0%). A similar pattern is exhibited by the *N*-benzylated analogues **5d** and **9d** (*Pf* viability: **5d**: 52%; **9d**: *ca*. 0%). However, changing the length of the hydrophobic patch has little effect on the significant levels of inhibitory activity exhibited by the mono-methylene-bridged ligand **5c** and the bi-methylene-bridged, *N*-benzylated analogue **9c** – both of which exhibit *ca*. 0% *Pf* viability at 20 μM and comparable IC_{50} values.

v) The four most active *N*-benzylated analogues each contain coordinating heteroatoms in their aryl (or heteroaryl) groups [**5b**: Ar = 3-(hydroxymethyl)-phenyl; **5c** and **9c**: Ar = 2-pyridyl; and **9d**: Ar = 3-hydroxyphenyl] – reflecting, perhaps, the importance of their hydrogen-bonding donor and/or acceptor capacity.

vi) The evident activity of the *N*-propynylated derivative containing a bi-methylene spacer **18b** raises the possibility of using Click Chemistry methodology to access more structurally diverse ligands as potential antimalarial agents.

The parasite is expected to possess the esterases necessary to hydrolyse the phosphonate esters and thus release the phosphonic acids, designed as *Pf*DXR-specific ligands. In order to establish whether the observed *Pf* parasite inhibition by the most active phosphonate esters was due to binding of their hydrolysed analogues to the *Pf*DXR active-site, *Pf*DXR enzyme inhibition assays of three of the corresponding phosphonic acids were undertaken. Surprisingly, 20 μM solutions of these compounds exhibited very little, if any, activity against *Pf* DXR (**10c** and **10d**: 0%; **19b**: 6.7%) compared to the known inhibitor FR900098 (73.3% inhibition). These results indicate that the phosphonate esters are inhibiting the *Pf* parasite *via* a different but, as yet, unknown mechanism – a conclusion supported by the unexpected SAR data discussed above.

In expectation that the synthetic ligands would bind to the *Pf*DXR receptor, *in silico* docking studies of selected benzylated and non-benzylated ligands were undertaken, following previously reported methods[9] but using a flexible docking approach. The *Pf*DXR crystal structure[11] was accessed from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank using discovery studio.[12] The best conformation obtained in each case appeared to correlate well with the active-site cavity of the *Pf*DXR crystal structure; as illustrated for the docking of phosphonic acid **10a** (Figure 4), potential hydrogen-bonding interactions can be identified while the benzyl group occupies a hydrophobic cavity and is appropriately orientated for π-stacking with the indole moiety of a proximate tryptophan residue. However, the bioassay data demand a different explanation for the observed activity of the phosphonate esters reported in this study. It is thus apparent that other factors, such as, ligand solvation and receptor hydration effects may need to be considered in future docking studies.

3. Conclusions

Sixteen *N*-benzylated and non-benzylated phosphonate esters have been synthesised as potential anti-malarial agents, six of which exhibit significant activity at low micromolar concentrations against the *Plasmodium falciparum* parasite. Although designed specifically as *Pf* DXR inhibitors, it is apparent from the bioassay data that a different mechanism of action is involved.

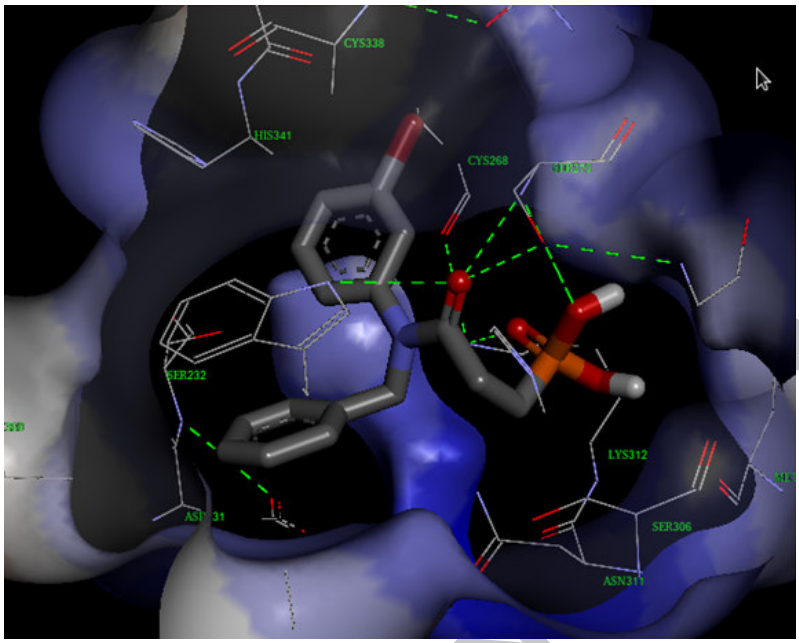


Figure 4. Docked conformation of phosphonic acid **10a** in the *Pf*DXR active-site (3AU9).[11] Protein active-site residues are shown in wire frame and the ligand in stick model, all coloured by atom type. Hydrogen–bonding interactions are shown as green dashed lines.

4. Experimental

4.1. General methods

NMR spectra were typically recorded on Bruker 300 or 400 MHz spectrometers in $CDCl_3$ and calibrated using solvent signals [δ_H: 7.26 ppm for residual $CHCl_3$. δ_C: 77.0 ppm ($CDCl_3$)]. Melting points were measured using a hot stage apparatus and are uncorrected. IR spectra were recorded neat on a Perkin Elmer Spectrum 100 FT-IR spectrometer with a diamond window. High-resolution mass spectra (HRMS) were recorded on a Waters API Q-TOF Ultima spectrometer (University of Stellenbosch, Stellenbosch, South Africa).

4.2. Synthesis

4.2.1. General procedure for the reaction of primary amines with benzyl bromide.

Compounds **5a-5e**, **6a-6e** and **7a-7e** are known[9,13,14] but a different synthetic approach to compounds **7a-7e**, illustrated by the following example, was used in this work. Benzyl bromide (1.90 mL, 16 mmol) was added to a mixture of 3-aminobenzyl alcohol **4b** (2.0 g, 16 mmol) and $NaHCO_3$ (1.36 g, 16 mmol) in dry THF (10 mL). The mixture was stirred at r.t. overnight under nitrogen. Water (20 mL) was then added and the aqueous layer extracted with $CHCl_3$ (2 × 50 mL). The combined organic extracts were then washed with brine (2 × 30 mL), dried with anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. Chromatography [PLC on silica gel; elution with hexane-EtOAc (5:1)] of the residue gave two fractions.

Fraction 1: *N*-Benzyl-3-(hydroxymethyl)aniline **7b** as a brown oil (2.17 g, 63%); ν_{max}/cm^{-1} 3385 (OH) and 3340 (NH); δ_H/ppm (400 MHz; $CDCl_3$) 2.87 (1H, s, NH), 4.21 (2H, s, NCH_2Ph), 4.46 (2H, s, CH_2OH), 6.45 (1H, d, *J* = 8.1 Hz, Ar-H), 6.52 (1H, s, Ar-H), 6.59 (1H, d, *J* = 7.51 Hz, Ar-H), 7.76 (1H, t, *J* = 7.76 Hz, Ar-H) and 7.16-7.28 (5H, overlapping m, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 48.3 (NCH_2Ph), 65.5 (CH_2OH), 111.4, 112.1, 116.2, 127.4, 127.6, 128.7, 129.6, 139.4, 142.2 and 148.5 (Ar-C).

Fraction 2: N-*Benzyl-3-[(benzyloxy)methyl]aniline* **11** as white crystals (1.08 g, 19%), m.p. 88-90 ^oC; [HRMS: *m/z* calculated for $C_{21}H_{22}NO$ (MH^+) 304.1701. Found 304.1698]; ν_{max}/cm^{-1} 3384 (NH), δ_H/ppm (400 MHz; $CDCl_3$) 4.57 (2H, s, NCH_2Ph), 4.66 [4H, s, $O(CH_2)_2$], 6.68 (1H, d, *J* = 7.41 Hz , Ar-H), 6.71 (1H, m, Ar-H), 6.78 (1H, br, s, NH), 7.16 (1H, m, Ar-H), 7.24-7.26 (7H, overlapping m, Ar-H), 7.30-7.33 (4H, overlapping m, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 46.3 (NCH_2Ph), 75.1 [2C, $O(CH_2)_2$] 111.7, 112.5, 115.9, 126.7, 127.0, 127.5, 128.2, 128.9, 129.1, 129.7, 136.2, 140.3, 141.5 and 146.8 (Ar-C).

4.2.2. General procedure for the preparation of the 3-chloropropanamides 8a-e.

N-Benzyl-3-bromoaniline **7a** (0.68 g, 2.2 mmol) was dissolved in dry THF (20 mL) and NaH (0.10 g, 4.2 mmol) was added in portions. The mixture was stirred for 30 min and 3-chloropropanoyl chloride (0.22 mL, 2.2 mmol) was then added slowly to control the evolution of hydrogen. After stirring overnight at r.t. under N_2, the solvent was removed *in vacuo*, and the crude product was extracted into EtOAc (2 × 50 mL). The extracts were combined, washed sequentially with satd. aq. $NaHCO_3$ (2 × 30 mL), distilled water (2 × 30 mL) and brine (2 × 30 mL), dried ($MgSO_4$) and filtered, and the solvent was removed *in vacuo*. Chromatography of the residue [PLC on silica gel; elution with hexane-EtOAc (7:3)]

afforded N-*benzyl-N-(3-bromophenyl)-3-chloropropanamide* **8a** as a yellow oil (0.45 g, 50%) [HRMS: *m/z* calculated for $C_{16}H_{16}NOClBr$ (MH^+) 352.0104. Found 352.0103]; ν_{max}/cm^{-1}: 1649 (C=O); δ_H/ppm (400 MHz; $CDCl_3$) 2.47 (2H, t, *J* = 6.53 Hz, CH_2CO), 3.74 (2H, t, *J* = 6.64 Hz CH_2Cl), 4.82 (2H, s, NCH_2Ph), 6.83 (1H, d, *J* = 7.66 Hz, Ar-H), 7.10-7.19 (4H, overlapping m, Ar-H), 7.20 (3H, overlapping m, Ar-H), and 7.39 (1H, d, *J* = 8.06 Hz, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 37.3 (CH_2CO), 40.1 (CH_2Cl) and 53.2 (NCH_2Ph), 123.1, 127.6, 127.8, 128.7, 128.9, 131.0, 131.7, 131.7, 136.8 and 143.1 (Ar-C) and 169.3 (C=O).

4.2.2.1. N-*Benzyl-3-chloro*-N-*[3-(hydroxymethyl)phenyl]propanamide* 8b as a pale yellow oil (0.27 g, 36%). [HRMS: *m/z* calculated for $C_{17}H_{18}NO_2Cl$ (MH^+) 304.1104. Found 304.1107]; ν_{max}/cm^{-1}: 3346 (OH) and 1649 (C=O); δ_H/ppm (300 MHz; $CDCl_3$) 2.47 (2H, t, *J* = 6.72 Hz, CH_2CO), 3.71 (2H, t, *J* = 6.73 Hz, CH_2Cl), 4.59 (2H, s, NCH_2Ph), 4.86 (2H, s, CH_2OH), 6.82 (1H, td, *J* = 2.14 and 4.57 Hz, Ar-H), 6.95 (1H, s, Ar-H), 7.11-7.19 (5H, overlapping m, Ar-H) and 7.24 (2H, m, Ar-H); δ_C/ppm (75 MHz; $CDCl_3$) 37.3 (CH_2CO), 40.3 (CH_2Cl), 53.3 (NCH_2Ph) and 64.5 (CH_2OH), 126.7, 127.5, 127.6, 127.7, 128.6, 128.9, 129.9, 137.2, 142.0 and 143.11 (Ar-C) and 169.6 (C=O).

4.2.2.2. N-*Benzyl-3-chloro*-N-*(2-pyridyl)propanamide* 8c as a yellow oil (0.28 g, 33%) [HRMS: *m/z* calculated for $C_{15}H_{15}ClN_2O$ (MH^+) 274.2738. Found 274.2735]; ν_{max}/cm^{-1}: 1661 (C=O); δ_H/ppm (400 MHz; $CDCl_3$) 2.82 (2H, t, *J* = 6.94 Hz, CH_2CO), 3.80 (2H, t, *J* = 6.94 Hz, CH_2Cl), 4.51 (2H, s, NCH_2Ph), 6.47 (1H, d, *J* = 8.74 Hz, Ar-H), 6.63 (1H, t, *J* = 6.33 Hz, Ar-H), 7.29-7.36 (5H, overlapping m, Ar-H), 7.54 (1H, ddd, *J* = 1.70, 7.19 and 8.79 Hz, Ar-H) and 7.92 (1H, d, *J* = 4.95 Hz, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 39.3 (CH_2CO), 40.3 (CH_2Cl) and 46.1 (NCH_2Ph), 118.0, 112.0, 126.9, 127.5, 128.8, 140.7, 141.7, 142.1 and 144.2 (Ar-C) and 17.4 (C=O).

4.2.2.3. N-*Benzyl-3-chloro*-N-*(3-hydroxyphenyl)propanamide* 8d as a brown oil (0.33 g, 39%) {[HRMS: *m/z* calculated for $C_{16}H_{17}NO_2Cl$ (MH^+) 290.0948. Found 290.0941]; ν_{max}/cm^{-1}: 3345 (OH) and 1668 (C=O); δ_H/ppm (400 MHz; $CDCl_3$) 2.82 (2H, t, *J* = 6.94 Hz, CH_2CO), 3.80 (2H, t, *J* = 6.94 Hz, CH_2Cl), 4.51 (2H, s, NCH_2Ph), 6.47 (1H, d, *J* = 8.74 Hz, Ar-H), 6.63 (1H, t, *J* = 6.33 Hz, Ar-H), 7.29-7.36 (5H, overlapping m, Ar-H), 7.54 (1H, ddd, *J* = 1.70, 7.19 and 8.79 Hz, Ar-H) and 7.92 (1H, d, *J* = 4.95 Hz, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 39.3 (CH_2CO), 40.3 (CH_2Cl) and 46.1 (NCH_2Ph), 118.0, 112.0, 126.9, 127.5, 128.8, 140.7, 141.7, 142.1 and 144.2 (Ar-C) and 17.4 (C=O); and the bis-acylated analogue,

*3-(*N-*Benzyl-3-chloropropanamido)phenyl 3-chloropropanoate* **12** as a brown oil (0.16 g, 14%) [HRMS: *m/z* calculated for $C_{19}H_{19}NO_3Cl_2$ (MH^+) 380.0820. Found 380.0823]; ν_{max}/cm^{-}

^{1}: 1727 (O-C=O) and 1668 (N-C=O); δ_H/ppm (400 MHz; $CDCl_3$) 2.58-2.66 (4H, m, 2 × CH_2CO), 3.77 (4H, m, 2 × CH_2Cl), 4.66 (2H, s, NCH_2Ph), 6.68 (2H, m, Ar-H), 7.03-7.15 (6H, overlapping m, Ar-H) and 7.29 (1H, t, *J* = 8.15 Hz, Ar-H); δ_C/ppm (100 Hz; $CDCl_3$) 36.5 and 37.4 (2 × *C*H_2CO), 40.5 and 42.1 (2 × CH_2Cl) and 49.9 (NCH_2Ph), 113.4, 116.2, 118.2, 127.7, 128.6, 129.9, 130.8, 137.1, 142.9 and 153.6 (Ar-C), 170.9 and 172.0 (2 × C=O).

4.2.2.4. N-*Benzyl-3-chloro*-N-*(3-nitrophenyl)propanamide* 8e as a pale yellow oil (0.48 g, 52%) [HRMS: *m/z* calculated for $C_{16}H_{16}N_2O_3Cl$ (MH^+) 319.0849. Found 319.0842]; ν_{max}/cm^{-1}: 1659 (C=O); δ_H/ppm (300 MHz; $CDCl_3$) 2.47 (2H, t, *J* = 6.43 Hz, CH_2CO), 3.76 (2H, t, *J* = 6.53 Hz, CH_2Cl), 4.89 (2H, s, NCH_2Ph), 7.09-7.12 (2H, overlapping m, Ar-H), 7.20-7.2 (4H, overlapping m, Ar-H), 7.47 (1H, t, *J* = 8.09 Hz, Ar-H), 7.84 (1H, s, Ar-H) and 8.13 (1H, d, *J* = 8.05 Hz, Ar-H); δ_C/ppm (75 MHz; $CDCl_3$) 37.4 (*C*H_2CO), 40.0 (CH_2Cl) and 53.2 (NCH_2Ph), 123.4, 123.8, 128.1, 128.4, 128.9, 130.7, 135.1, 136.2, 142.8 and 148.9 (Ar-C) and 169.1 (C=O).

4.2.3. General procedure for Michaelis-Arbuzov reactions of *N*-benzyl-3-chloro-propanamides 9a-e.

A mixture of *N*-benzyl-*N*-(3-bromophenyl)-3-chloropropanamide **7a** (0.25 g, 0.72 mmol) and triethyl phosphite (0.25 mL, 1.44 mmol) was heated under reflux at 120-150 ^{o}C for 6 h under N_2. The reaction was monitored by TLC and, upon completion of the reaction, the crude product was chromatographed [PLC on silica gel; elution with EtOAc] to afford *diethyl 2-[*N-*benzyl*-N-*(3-bromophenyl)carbamoyl]ethylphosphonate* **9a** as a yellow oil (0.16 g, 51%) [HRMS: *m/z* calculated for $C_{20}H_{26}NO_4BrP$ (MH^+) 454.0763. Found 454.0777]; ν_{max}/cm^{-1}: 1654 (C=O) and 1240 (P=O); δ_H/ppm (300 MHz; $CDCl_3$) 1.18 (6H, t, *J* = 7.06 Hz, 2 × CH_3), 2.03 (2H, m, CH_2P), 2.25 (2H, m, CH_2CO), 3.96 (4H, m, 2 × O*C*H_2CH_3), 4.79 (2H, s, NCH_2Ph), 6.82 (1H, d, *J* = 7.53 Hz, Ar-H), 7.09-7.13 (4H, overlapping m, Ar-H), 7.19 (3H, overlapping m, Ar-H) and 7.40 (1H, d, *J* = 7.93 Hz, Ar-H); δ_C/ppm (75 MHz; $CDCl_3$) 16.5 (d, J_{P-C} = 6.02 Hz, 2 × CH_3), 20.9 (d, J_{P-C} = 143.67 Hz, CH_2P), 27.9 (*C*H_2CO), 53.5 (NCH_2Ph), 61.8 (d, J_{P-C} = 6.44 Hz, 2 × O*C*H_2CH_3), 123.1, 127.5, 127.8, 128.7, 128.9, 131.1, 131.2, 131.7, 136.9 and 143.2 (Ar-C) and 170.7 (C=O).

4.2.3.1. *Diethyl 2-{*N-*benzyl*-N-*{[3-(hydroxymethyl)phenyl]carbamoyl}ethylphosphonate* 9b as a yellow oil (0.15 g, 47%) following flash chromatography (on silica gel; elution with EtOAc) [HRMS: *m/z* calculated for $C_{21}H_{27}NO_5P$ $(M-1)^+$ 404.1625. Found 404.1627]; ν_{max}/cm^{-1}: 3345 (OH), 1664 (C=O) and 1245 (P=O); δ_H/ppm (400 MHz; $CDCl_3$) 1.25 (6H, t, *J*

= 6.98 Hz, 2 × CH_3), 2.12 (2H, m, CH_2P), 2.34 (2H, m,CH_2CO), 4.01 (4H, m, 2 × O*C*H_2CH_3), 4.65 (2H, s, NCH_2Ph), 4.85 (2H, s, C*H*$_2$OH), 6.89 (1H, d, *J* = 7.45 Hz, Ar-H), 7.07 (1H, s, Ar-H), 6.92 (1H, d, *J* = 7.08 Hz, Ar-H), 7.19 (2H, d, *J* = 3.6 Hz), 7.24-7.29 (6H, overlapping m, Ar-H and OH) and 7.07 (1H, d, *J* = 7.60 Hz, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 16.5 (d, J_{P-C} = 6.02 Hz, 2 × CH_3), 20.4 (d, J_{P-C} = 143.22 Hz, CH_2P), 29.9 (*C*H_2CO), 53.4 (NCH_2Ph), 61.9 (d, *J*= 6.44 Hz, 2 × O*C*H_2CH_3), 64.6 (CH_2OH), 122.7, 126.7, 126.9, 127.0, 127.6, 127.7, 128.6, 128.9, 129.9, 131.4, 131.6 and 135.3 (Ar-C) and 163.7 (C=O).

4.2.3.2. ***Diethyl 2-[*N*-benzyl-*N*-(2-pyridyl)carbamoyl]ethylphosphonate*** **9c** as a yellow oil (0.14 g, 49%) [HRMS: *m/z* calculated for $C_{19}H_{26}NO_4P$ (MH^+) 377.1630. Found 377.1627]; ν_{max}/cm^{-1}: 1670 (C=O) and 1248 (P=O); δ_H/ppm (400 MHz; $CDCl_3$) 1.27 (6H, t, *J* = 6.45 Hz, 2 × CH_3), 2.17 (2H, m, CH_2P), 2.58 (2H, m, CH_2CO), 4.06 (4H, m, 2 × O*C*H_2CH_3), 5.10 (2H, s, NCH_2Ph), 7.22-7.31 (7H, overlapping m, Ar-H), 7.67 (1H, td, *J* = 1.92 and 7.77 Hz, Ar-H) and 8.52 (1H, d, *J* = 3.66 Hz, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 16.5 (d, J_{P-C} = 6.06 Hz, 2 × CH_3), 21.7 (d, J_{P-C} = 143.57 Hz, CH_2P), 28.3 (*C*H_2CO), 51.7 (NCH_2Ph), 61.6 (d, J_{P-C} = 6.42 Hz, 2 × O*C*H_2CH_3), 121.9, 122.5, 127.5, 127.8, 128.0, 128.1, 128.6, 135.1, 137.4, 138.6 and149.4 (Ar-C) and 155.0 (C=O).

4.2.3.3. ***Diethyl 2-[*N*-benzyl-*N*-(3-hydroxyphenyl)carbamoyl]ethylphosphonate*** **9d** as a yellow oil (0.21 g, 51%) [HRMS: *m/z* calculated for $C_{20}H_{27}NO_5P$ (MH^+) 392.1627. Found 392.1623]; ν_{max}/cm^{-1}: 3340 (OH), 1667 (C=O) and 1242 (P=O); δ_H/ppm (400 MHz; $CDCl_3$) 1.28 (6H, t, *J* = 6.98 Hz, 2 × CH_3), 2.12 (2H, m, CH_2P), 2.43 (2H, m, CH_2CO), 4.03 (4H, m, 2 × O*C*H_2CH_3), 4.86 (2H, s, NCH_2Ph), 6.45 (1H, d, *J* = 7.74 Hz, Ar-H), 6.54 (1H, s, Ar-H), 6.77 (1H, dd, *J* = 1.68 and 8.17 Hz, Ar-H), 7.11 (1H, t, *J* = 8.01 Hz, Ar-H) and 7.21-7.24 (5H, overlapping m, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 16.4 (d, J_{P-C} = 6.20 Hz, 2 × CH_3), 21.6 (d, J_{P-C} = 143.44 Hz, CH_2P), 27.3 (*C*H_2CO), 53.5 (NCH_2Ph), 62.2 (d, J_{P-C} = 6.43 Hz, 2 × O*C*H_2CH_3), 115.5, 115.7, 119.3, 127.5, 128.5, 128.9, 130.4, 137.5, 142.8 and 158.2 (Ar-C) and 171.1 (C=O).

4.2.3.4. ***Diethyl 2-[*N*-benzyl-*N*-(3-nitrophenyl)carbamoyl]ethylphosphonate*** **9e** as a yellow oil (0.35 g, 59%) [HRMS: *m/z* calculated for $C_{20}H_{26}N_2O_6P$ (MH^+) 421.1526. Found 421.1522]; ν_{max}/cm^{-1}: 1661 (C=O) and 1243 (P=O); δ_H/ppm (400 MHz; $CDCl_3$) 1.18 (6H, t, *J* = 6.98 Hz, 2 × CH_3), 2.06 (2H, m, CH_2P), 2.25 (2H, m, CH_2CO), 3.96 (4H, m, 2 × O*C*H_2CH_3), 4.86 (2H, s, NCH_2Ph), 7.09-7.26 (6H, overlapping m, Ar-H), 7.46 (1H, t, *J* = 8.04 Hz, Ar-H), 7.84 (1H, s, Ar-H) and 8.12 (1H, d, *J* = 7.46 Hz, Ar-H); δ_C/ppm (100 MHz; $CDCl_3$) 16.4 (d, J_{P-C} = 6.20 Hz, 2 × CH_3), 21.8 (d, J_{P-C} = 143.8 Hz, CH_2P), 28.0 (*C*H_2CO),

53.4 (NCH_2Ph), 61.8 (d, J_{P-C} = 6.42 Hz, 2 × OCH_2CH_3), 123.3, 123.7, 128.0, 128.4, 128.8, 130.7, 135.1, 136.3, 142.9 and 148.9 (Ar-C) and 170.7 (C=O).

4.2.4. Preparation of the *O*-benzylated analogues 13 and 14.

To a solution of diethyl [*N*-(3-hydroxyphenyl)carbamoyl]ethylphosphonate **6d** (0.35 g, 1.2 mmol) dry THF (5mL), NaH (0.030 g, 1.3 mmol) was added in portions, followed by benzyl bromide (0.19 mL, 1.5 mmol) in dry THF (10 mL). The mixture was refluxed for 4 h., and the crude product was flash chromatographed [on silica gel; elution with hexane-EtOAc (3:2)] to afford two fractions.

Fraction 1: *Diethyl 2-{[3-(benzyloxy)phenyl]carbamoyl}ethylphosphonate* **13** as a yellow oil (0.077 g, 16%). [HRMS: *m/z* calculated for $C_{20}H_{27}NO_5P$ (MH^+) 329.1627. Found 392.1616]; ν_{max}/cm^{-1}: 1647 (C=O); δ_H/ppm (300 MHz; $CDCl_3$) 1.30 (6H, t, *J* = 1.31 Hz, 2 × CH_3), 2.18 (2H, m, CH_2P), 2.71 (2H, d, *J* = 6.19 Hz, CH_2CO), 4.11 (4H, m, 2 × OCH_2CH_3), 5.05 (2H, s, OCH_2Ph), 6.69 (1H, d, *J* = 7.35 Hz, Ar-H), 7.11-7.48 (8H, overlapping m, Ar-H) and 9.07 (1H, s, NH); δ_C/ppm (75 MHz; $CDCl_3$) 16.5 (d, J_{P-C} = 5.73 Hz, 2 × CH_3), 21.1 (d, J_{P-C} = 143.7 Hz, CH_2P), 30.2 (CH_2CO), 62.3 (d, J_{P-C} = 6. 32 Hz, 2 × OCH_2CH_3), 70.1 (OCH_2Ph), 106.5, 110.8, 112.3, 127.6, 128.0, 128.6, 128.8, 129.7, 137.2, 140.0 and 159.5 (Ar-C) and 169.4 (C=O).

Fraction 2: *Diethyl* N-*benzyl-{[3-(benzyloxy)phenyl]carbamoyl}ethylphosphonate* **14** as a yellow oil (0.27 g, 46%) [HRMS: *m/z* calculated for $C_{27}H_{33}NO_5P$ (MH^+) 482.2096. Found 482.2094]; ν_{max}/cm^{-1}: 1645 (C=O); δ_H/ppm (300 MHz; $CDCl_3$) 1.13 (6H, t, *J* = 6.37 Hz, 2 × CH_3), 1.98 (2H, m, CH_2P), 2.25 (2H, m, CH_2CO), 3.92 (4H, m, 2 × OCH_2CH_3), 4.73 (2H, s, CH_2Ph), 4.84 (2H, s, OCH_2Ph), 6.46 (2H, s, Ar-H), 6.82 (1H, d, *J* = 8.57 Hz, Ar-H) and 7.05-7.26 (11H, overlapping m, Ar-H); δ_C/ppm (75 MHz; $CDCl_3$) 16.5 (d, J_{P-C} = 6.01 Hz, 2 × CH_3), 21.6 (d, J_{P-C} = 142.7 Hz, CH_2P), 27.7 (CH_2CO), 53.4 (NCH_2Ph), 61.7 (d, J_{P-C} = 6.36 Hz, 2 × OCH_2CH_3), 70.4 (OCH_2Ph), 114.9, 115.2, 121.0, 127.6, 127.7, 128.3, 128.5, 128.8, 129.0, 130.5, 136.5 and 137.4 (Ar-C) and 159.8 (C=O).

4.2.5. Tert-*butyldimethylsilyl ether-protected phosphonate ester* 15. To a solution of diethyl [*N*-(3-hydroxyphenyl)carbamoyl]methylphosphonate **5d** (0.50 g, 1.7 mmol) and imidazole (0.15 g, 2.3 mmol) in dichloromethane (20 mL) at 0 ^oC (ice bath) was added *tert*-butyldimethylsilyl chloride (0.29 g, 1.9 mmol) in portions, and the mixture was stirred at r.t. for 2 h. The mixture was diluted with DCM (10 mL) and washed with water (2 × 20 mL) and

brine (2 × 20 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography [on silica gel; elution with hexane-EtOAc (9:1)] to afford the *tert*-butyldimethylsilyl ether-protected phosphonate ester **15** as a white solid (0.62 g, 88%), m.p. 70-72 °C [HRMS: *m/z* calculated for $C_{18}H_{33}NO_5PSi$ (MH^+) 402.1866. Found 402.1861]; ν_{max}/cm^{-1}: 1673 (C=O); δ_H/ppm (400 MHz; $CDCl_3$) 0.20 [6H, s, $Si(CH_3)_2$], 0.97 [9H, s, $C(CH_3)_3$], 1.35 (6H, t, J = 7.07 Hz, 2 × CH_3), 2.98 (2H, d, J_{P-H} = 21.0 Hz, CH_2P), 4.17 (4H, m, 2 × OCH_2CH_3), 6.60 (lH, dd, J =1.32 and 7.96 Hz, Ar-H), 7.06 (lH, dd, J = 1.50 and 6.58 Hz, Ar-H), 7.16 (2H, m, Ar-H) and 8.71 (lH, s, NH); δ_C/ppm (100 MHz; $CDCl_3$) -4.3 [$Si(CH_3)_2$], 16.5 (d, J_{P-C} = 6.03 Hz, 2 × CH_3), 18.3 [$C(CH_3)_3$], 25.8 [$C(CH_3)_3$], 36.3 (d, J_{P-C} = 129.4 Hz, CH_2P), 63.2 (d, J_{P-C} = 6.44 Hz, 2 × OCH_2CH_3), 112.0, 112.9, 116.3, 129.7, 138.9 and 156.3 (Ar-C) and 162.0 (C=O).

4.2.6. Preparation of the acylated propargylamine derivatives 17a,b is illustrated by the following example. A solution of propargylamine **16** (0.58 mL, 9.0 mmol) and triethylamine (1.3 mL, 9.0 mmol) in dry THF (20 mL) was cooled to 0 °C (ice-bath) under N_2. Chloroacetyl chloride (0.72 mL, 9.0 mmol) was then added slowly through a septum to the solution which was kept at 0 °C for 45 min and then stirred for 2 h at r.t. The solvent was removed *in vacuo* and the residue was dissolved in DCM (2×50 mL). The resulting solution was washed sequentially with 10% dil HCl (50 mL) and water (50 mL); the aqueous layers were then re-extracted with DCM (100 mL). The organic layers were combined, dried with anhydrous $MgSO_4$ and evaporated *in vacuo* to afford *2-chloro*-N-*(2-propynyl)acetamide* **17a** as an off-white solid (1.13 g, 95%), m.p. 68-70 °C [HRMS: *m/z* calculated for C_5H_6NOCl (MH^+) 131.0142. Found 131.0132]; ν_{max}/cm^{-1}: 3387 (NH) and 1661 (C=O); δ_H/ppm (400 MHz; $CDCl_3$) 2.28 (1H, t, J = 2.57 Hz, CH), 4.07 (2H, s, CH_2Cl), 4.11 (2H, m, NCH_2) and 6.83 (1H, s, NH); δ_C/ppm (100 MHz; $CDCl_3$) 29.7 (CH_2N), 42.5 (CH_2Cl), 72.3 (C≡*C*H), 78.7 (*C*≡CH) and 165.8 (C=O).

4.2.6.1. ***3-Chloro*-N-*(2-propynyl)propanamide*** **17b** (obtained using propanoyl chloride) as a white solid (1.21 g, 92%), m.p. 72-74 °C [HRMS: *m/z* calculated for C_6H_8NOCl (MH^+) 146.0367. Found 146.0373]; ν_{max}/cm^{-1}: 3377 (NH) and 1653 (C=O); δ_H/ppm (400 MHz; $CDCl_3$) 2.25 (1H, t, J = 2.55 Hz, CH), 2.66 (2H, t, J = 6.49 Hz, CH_2CO), 3.81 (2H, t, J = 6.49 Hz, CH_2Cl), 4.09 (2H, m, NCH_2) and 5.83 (1H, s, NH); δ_C/ppm (100 MHz; $CDCl_3$) 29.5 (CH_2N), 39.4 (*C*H_2CO), 39.9 (CH_2Cl), 72.1 (C≡*C*H), 79.2 (*C*≡CH) and 169.2 (C=O).

4.2.7. Phosphonation of propargylamine derivatives 18a,b *via* the Arbuzov reaction is illustrated by the following example. Triethyl phosphite (2.3 mL, 14 mmol) was added through a septum to 2-chloro-*N*-(2-propynyl)acetamide **17a** (0.9 g, 7 mmol) under N_2 in an oven-dried, round-bottomed flask and the resulting mixture was refluxed for 4 h. The cooled mixture was stirred with hexane (5 × 25 mL), decanting the hexane layer each time to remove excess triethyl phosphite. Flash chromatography [on silica gel; elution with hexane-EtOAc (1:4)] and evaporation of the solvent *in vacuo* afforded *diethyl [*N*-(2-propynyl)carbamoyl]-methylphosphonate* **18a** as a brown oil (1.08 g, 68%) [HRMS: *m/z* calculated for $C_9H_{17}NO_4P$ (MH^+) 234.0895. Found 234.0892]; ν_{max}/cm^{-1}: 3340 (NH), 1734 (C=O) and 1245 (P=O); δ_H/ppm (400 MHz; $CDCl_3$) 1.34 (6H, t, J = 7.07 Hz, 2 × CH_3), 2.21 (1H, t, J = 2.25 Hz, CH), 2.86 (2H, d, J = 20.58 Hz, CH_2P), 4.05 (2H, dd, J = 2.54 and 5.34, CH_2N), 4.16 (4H, m, 2 × OCH_2CH_3) and 7.10 (1H, s, NH); δ_C/ppm (100 MHz; $CDCl_3$) 16.5 (d, J = 6.11 Hz, 2 × CH_3), 29.6 (CH_2N), 35.0 (d, J_{P-C} = 130.9 Hz, CH_2P), 63.08 (d, J_{P-C} = 6.50 Hz, 2 × OCH_2CH_3), 71.7 (C≡*C*H), 79.2 (*C*≡CH) and 163.9 (C=O).

4.2.7.1. ***Diethyl [*N*-(2-propynyl)carbamoyl]ethylphosphonate*** **18b** (obtained from compound **17b**) as a dark brown oil (1.11 g, 65%) [HRMS: *m/z* calculated for $C_{10}H_{19}NO_4Cl$ (MH^+) 248.1052. Found 248.1051]; ν_{max}/cm^{-1}: 3340 (NH), 1738 (C=O) and 1247 (P=O); δ_H/ppm (400 MHz; $CDCl_3$) 1.29 (6H, t, J = 7.07 Hz, 2 × CH_3), 2.07 (2H, m, CH_2P), 2.18 (1H, t, J = 2.56 Hz, CH), 2.49 (2H, m, CH_2CO), 4.01 (2H, m, CH_2N), 4.07 (4H, m, 2 × OCH_2CH_3) and 7.13 (1H, s, NH); δ_C/ppm (100 MHz; $CDCl_3$) 16.5 (d, J_{P-C} = 6.06 Hz, 2 × CH_3), 21.0 (d, J_{P-C} = 143.7 Hz, CH_2P), 28.8 (CH_2CO), 29.3 (CH_2N), 61.6 (d, J_{P-C} = 6.57 Hz, 2 × OCH_2CH_3), 71.3 (C≡*C*H), 79.8 (*C*≡CH) and 170.9 (C=O).

4.3. Bioassay procedures.

4.3.1. *P.falciparum* pLDH assay. *P. falciparum* strain 3D7 parasites were cultured at 37 °C in RPMI 1640 media supplemented with 25 mM HEPES, 5% (w/v) Albumax II, 22 mM glucose, 0.65 mM hypoxanthine, 0.05 mg/mL gentamicin and 2-4% (v/v) human erythrocytes in flasks suffused with a 5% CO_2, 5% O_2, 90% N_2 gas mixture. For compound assays, cultures were diluted to a final concentration of 1% (v/v) erythrocytes and 2% parasitaemia and incubated with 3-fold serial dilutions (for IC_{50} determination) or a 20 µM final concentration of the compounds in 96-well plates (200 µL final volume) at 37 °C for 48 h. Following the incubation, residual parasite viability in individual wells was determined using a colorimetric parasite lactate dehydrogenase (pLDH) assay – 20 sµL was removed from the

culture wells and transferred to a duplicate 96-well plate containing 125 µL per well pLDH assay reagent (44 mM Tris buffer, pH 9, containing 0.18 M L-lactic acid, 0.13 mM acetylpyridine adenine dinucleotide, 0.39 mM nitrotetrazolium blue chloride, 0.048 mM phenazine ethosulphate and 0.16% (v/v) Triton X-100) and incubated at ambient temperature for 10 – 30 minutes. Colour development was measured as absorbance at 620 nm and the absorbance values were used to calculate percentage viability relative to control wells containing untreated parasite cultures. IC_{50} values for individual compounds were determined by non-linear regression of log (concentration) vs. percentage parasite viability plots using GraphPad Prism (v. 5.02).

4.3.2. DXR inhibition assay. Assays were conducted in a reaction mixture containing 100 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 0.3 mM NADPH, 0.3 mM DOXP and 20 µg/ml *Pf*DXR in a total volume of 200 µL. Samples were incubated at 37 ^oC, for 5 minutes before the reaction was initiated by adding DOXP substrate. The decrease in absorbance at 340 nm due to the decreasing concentration of NADPH (E_{NADPH} = 6.3 x 10^3 L/mol/cm) was followed for 10 min at 37 ^oC using a Synergy MX (BioTek®) thermostated spectrophotometer. Reactions were blanked against a reaction lacking the DOXP substrate. Enzyme activity in the absence of inhibitor was deemed to be 100% (*i.e.* 0% inhibition) and the % inhibition, which was determined in triplicate, was calculated relative to this. DXR activity was expressed in units per mg of protein where 1 unit is defined as the amount of enzyme that causes oxidation of 1 µmol of NADPH per minute.

4.3.3. Cell toxicity assay. HeLa cells were plated in 96-well plates to a final density $2x10^4$ cells/well and incubated overnight in culture medium (DMEM supplemented with 10% foetal bovine serum and penicillin/streptomycin/amphotericin B) in a CO_2 incubator at 37 ^oC. Compounds were added to the wells to a final concentration of 20 µM and incubation was continued for 24 h. A resazurin-based toxicology reagent (Sigma-Aldrich) was added (20 µL/well) and incubated for a further 2-4 h, after which cell viability was assessed by measuring fluorescence intensity (exc. 560 nm, em. 590 nm) in a Molecular Devices Spectramax M3 plate reader. Percentage cell viability was calculated for individual compound-treated wells from the fluorescence intensity readings relative to those obtained from control wells containing untreated cells.

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