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α-Phenylethyl Substituted Bis(pivaloyloxymethyl) Ester Analogues of Fosmidomycin and FR900098

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α-Phenylethyl substituted bis(pivaloyloxymethyl) ester analogues of the natural products Fosmidomycin and FR900098 have been synthesized, and their in vitro antimalarial activity determined. The α-phenylethyl substituted Fosmidomycin analogue displays moderate in vitro antimalarial activity against the chloroquine-sensitive strain 3D7 of *Plasmodium falciparum*.

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Introduction

According to estimations by the World Health Organisation, 1.5 to 2.7 million people die of malaria every year. Since the widespread resistance of *Plasmodium falciparum*, the causative agent of Malaria tropica, complicates an efficient therapy, novel antimalarial drugs based on new modes of action are urgently needed. A promising metabolic pathway for the development of novel antimalarials, antibiotics, and herbicides represents the DOXP/MEP pathway of isoprenoid biosynthesis, which is, for instance, present in *Plasmodium falciparum*, several pathogenic bacteria, and higher plants, but is absent in humans.[1] The natural products Fosmidomycin **1** and FR900098 **2** (Fig. 1) are potent inhibitors of the 1-desoxy-D-xylulose-5-phosphate reductoisomerase (DXR), a key enzyme of the DOXP/MEP pathway.[2] Currently Fosmidomycin is in clinical phase II trials in combination with the lincosamide antibiotic Clindamycin for the treatment of Malaria tropica.[3] However, a drawback of Fosmidomycin represents its relatively poor oral bioavailability of approximately 30%.[4] Furthermore, most phosphonohydroxamic acids prepared so far are of a hygroscopic nature, which makes their use and analysis difficult.[5]

In previous studies, we and other groups[5] have discovered that the phosphonic acid moiety of the lead compounds **1** and **2** as well as their hydroxamic acid functionality are essential for distinct antimalarial activity.[5–7] Moreover, Schlitzer and coworkers have shown that prodrugs of FR9000098, a more active analogue of Fosmidomycin, display improved in vivo antimalarial activity against *Plasmodium vinckei* in the mouse model.[7] Recently, we reported that α-phenylfosmidomycin **3** (IC_{50} 0.4 μM) exhibits potent activity against the chloroquine-resistant strain Dd2 of *Plasmodium falciparum*.[8a,b] Later, Van Calenbergh et al. confirmed the high antimalarial activity of α-aryl substituted Fosmidomycin analogues.[9] Furthermore, Schlitzer and our group have discovered that the hygroscopicity of phosphonohydroxamic acids can be overcome by masking the phosphonic acid moiety as a bis(pivaloyloxymethyl) ester.[6,7] We have shown that the non-hygroscopic bis(pivaloyloxymethyl) ester of **3** displays an IC_{50} value of 0.6 μM towards the chloroquine-sensitive strain 3D7 of *Plasmodium falciparum* and is approximately as active as the bis(pivaloyloxymethyl) ester of FR900098 **2**.[5] In addition, α-arylmethyl substituted Fosmidomycin analogues recently prepared in our group display antimalarial activity in the micromolar range.[5]

In order to expand the general knowledge of the structure–activity relationships, and to overcome the relatively poor bioavailability and the hygroscopicity of Fosmidomycin, we now describe the synthesis and antimalarial activity of α-phenylethyl substituted bis(pivaloyloxymethyl)ester analogues of the lead compounds **1**–**3**.

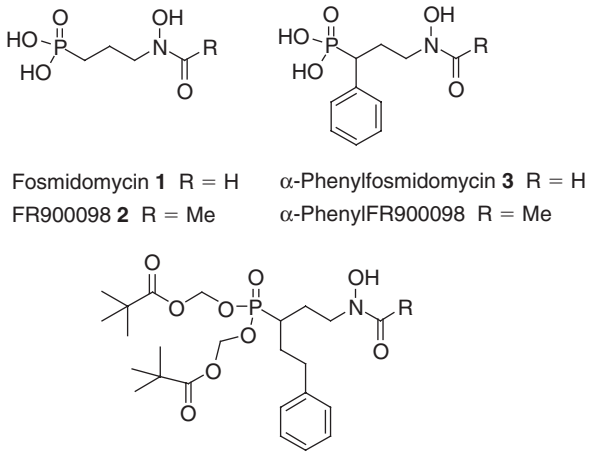

Fig. 1. Fosmidomycin and analogues.

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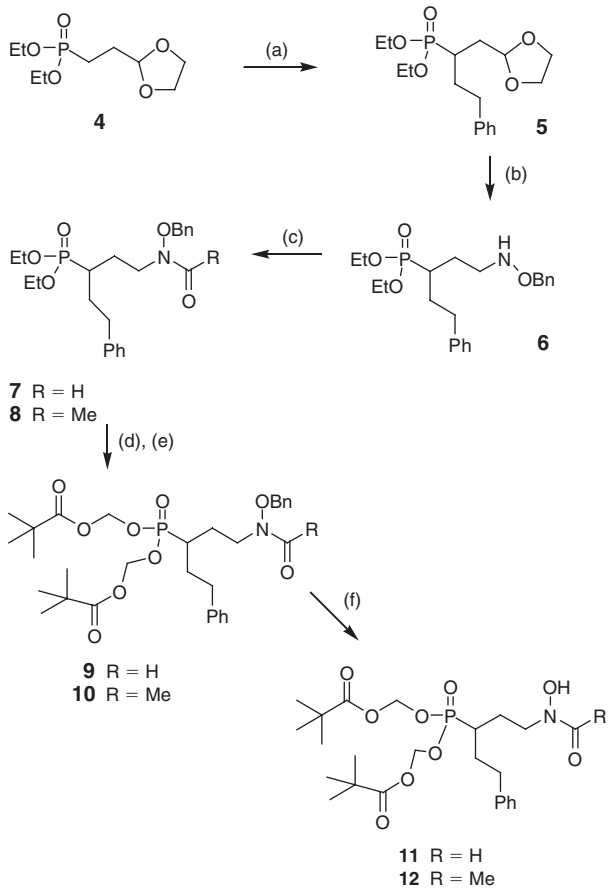

Scheme 1. Synthesis of α-phenylfosmidomycin analogues. *Reagents*: (a) Bu^nLi, $Ph(CH_2)_2Cl$; (b) HCl, $BnONH_2$, $NaCNBH_3$; (c) $HOOCH/Ac_2O$ or Ac_2O; (d) TMSBr, THF, H_2O; (e) $ClCH_2OOCC(CH_3)_3$, Et_3N; (f) H_2, Pd–C, MeOH.

Results and Discussion

Chemistry

Starting material **4** was prepared according to a literature procedure.[10] Synthesis of the target compounds **11** and **12** was conducted as reported previously for the preparation of α-hydroxymethyl- and α-arylmethyl-substituted Fosmidomycin analogues.[5]

Alkylation of dioxolane **4** with 2-phenylethyl chloride in presence of Bu^nLi in dry toluene afforded the corresponding phenylethyl substituted dioxolane **5**. The *O*-benzyl-protected hydroxylamine **6** was obtained by dioxolane hydrolysis, oximation, and subsequent reduction of the intermediate oximes with sodium cyanoborohydride in acidic medium. Acetylation or formylation of **6** led to protected formo- and aceto-hydroxamic acids **7** and **8**. Treatment of **7** and **8** with bromotrimethyl silane and water furnished the corresponding hygroscopic phosphonic acids, which were directly transformed into their bis(pivaloyloxymethyl)esters **9** and **10** by alkylation with chloromethyl pivalate in dry DMF. Finally, the *O*-benzyl group was removed in almost quantitative yields by catalytic hydrogenation to give the novel analogues **11** and **12** (Scheme 1). All compounds were characterized by elemental analysis or mass spectrometry, and IR, ^1H NMR, and ^{13}C NMR spectroscopy.

Table 1. Inhibition of *P. falciparum* growth [%] at 100, 10, and 1 μM

Compound no.	Inhibition of *P. falciparum* growth [%][A]		
	100 μM	10 μM	1 μM
3	100	89	65
11	100	42	4
12	100	13	4

[A] Mean values of two independent determinations.

Biological Activity

The in vitro antimalarial activity of compounds **11** and **12** was evaluated by an 8-[^{3}H]hypoxanthine incorporation assay according to the method of Desjardins using the chloroquine-sensitive strain 3D7 of *Plasmodium falciparum*.[11] Inhibition of *Plasmodium falciparum* growth at 100, 10, and 1 μM has been determined. The bis(pivaloyloxymethyl) ester of α-phenylfosmidomycin **3** was used as reference compound (Table 1). During the determination the bis(pivaloyloxymethyl) esters were converted into phosphonic acids by non-specific esterases.

The replacement of the phenyl nucleus of **3** by a phenylethyl group led to a significant reduction of antimalarial activity. In contrast to the higher activity of FR900098 compared to Fosmidomycin the formyl derivative **11** is more active than the acetyl derivative **12**.[5–7]

Conclusions

This paper reports the structural modification of α-phenylfosmidomycin **3**, a synthetic and highly active analogue of Fosmidomycin **1**. The phenyl nucleus of **3** was successfully replaced by a phenylethyl group. Due to their hygroscopicity the phosphonic acids were converted into stable, non-hygroscopic bis(pivaloyloxymethyl) esters. However, the new analogues **11** and **12** are significantly less active than the lead compounds **1**–**3**. As observed in case of other α-substituted analogues previously prepared in our group, the formyl derivative **11** was more active than the acetyl derivative **12**.[5] Since the bis(pivaloyloxymethyl) esters are converted into the corresponding free phosphonic acids by esterases during the assay, no animal experiments have been necessary.

Experimental

Elemental analyses were carried out with a Heraeus CHN-O-Rapid instrument. IR spectra were recorded on a Shimadzu FT-IR 8300. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker AMX 400 spectrometer using tetramethylsilane as internal standard and d_6-DMSO or $CDCl_3$ as solvents. Mass spectra were recorded on a Micromass VG 70–250S mass spectrometer (HRFAB), a Finnigan MAT 311 A mass spectrometer (EI), or a Varian MS 1200L mass spectrometer (ESI).

Chemistry

*General Procedure for the Preparation of (1-[1,3]Dioxolan-2-ylmethyl-3-phenylpropyl)-phosphonic Acid Diethyl Ester **5***

A solution of compound **4** (20 mmol) in dry toluene (30 mL) was cooled to −78°C, treated with *n*-butyllithium (20 mmol, 2.7 M solution in heptane), and stirred for 1 h. 2-Phenylethyl chloride (30 mmol) was added in one portion and the mixture

was allowed to warm to room temperature. After being stirred overnight, an aqueous solution of NH_4Cl (10%, 100 mL) was added, and the product extracted with EtOAc (2 × 50 mL). The organic layer was dried over Na_2SO_4, evaporated, and the resulting residue purified by column chromatography on silica gel (EtOAc) to give compound **5** as colourless oil (56%). Calc. for $C_{17}H_{27}NO_5P$ $[M+H]^+$: 343.1674. Found: 343.1667. ν_{max} (KBr)/cm^{-1} 1240 (P=O). δ_H (d_6-DMSO) 7.30–7.26 (m, 2H), 7.20–7.16 (m, 3H), 4.97 (t, 1H, *J* 5.1), 4.03–3.95 (m, 4H), 3.90–3.84 (m, 2H), 3.79–3.73 (m, 2H), 2.76–2.63 (m, 2H), 1.99–1.67 (m, 5H), 1.23 and 1.22 (2t, 6H, *J* 7.1). δ_C (d_6-DMSO) 142.04, 128.68, 128.66, 126.19, 102.61 (d, $^2J_{CP}$ 11.7), 64.63, 64.54, 61.48 (d, $^2J_{CP}$ 6.6), 33.21 (d, $^3J_{CP}$ 7.6), 32.78 (d, $^2J_{CP}$ 3.1), 31.09 (d, $^1J_{CP}$ 138.9), 30.93 (d, $^2J_{CP}$ 4.1), 16.67 (d, $^2J_{CP}$ 5.6).

General Procedure for the Preparation of (3-Benzyloxyamino-1-phenethylpropyl)phosphonic Acid Diethyl Ester ***6***

The acetal **5** (30 mmol) was refluxed with water (40 mL), HCl (1 mL, 37%), and acetone (100 mL) for 90 min. After the mixture was cooled to room temperature, the acetone was removed, water (200 mL) was added, and the mixture extracted with dichloromethane (3 × 100 mL). The organic layer was dried over Na_2SO_4 and then evaporated.

A solution of the remaining aldehyde in MeOH (40 mL) was treated with *O*-benzylhydroxylamine (30 mmol) and stirred at room temperature for 1 h. MeOH (430 mL) was then added and the resulting solution treated with $NaBH_3CN$ (90 mmol). HCl (37%, 30 mL) was added dropwise over 30 min under ice cooling. The mixture was allowed to warm to room temperature, and treated with additional $NaBH_3CN$ (20 mmol). After an total time of 2 h, the solution was concentrated, and aqueous KOH (10%) was added under ice cooling until an alkaline reaction was observed. The aqueous solution was extracted three times with CH_2Cl_2 (50 mL). The organic layers were combined, dried with $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel (95:5 EtOAc/MeOH) to give compound **6** as a colourless oil (78%). Found: C 65.0, H 8.0, N 3.7. $C_{22}H_{32}NO_4P$ requires C 65.2, H 8.0, N 3.5%. ν_{max} (KBr)/cm^{-1} 3243 (N–H), 1235 (P=O). δ_H (d_6-DMSO) 7.35–7.25 (m, 7H), 7.19–7.16 (m, 3H), 6.63 (t, 1H, *J* 5.9), 4.59 (s, 2H), 4.03–3.93 (m, 4H), 2.94–2.82 (m, 2H), 2.74–2.60 (m, 2H), 1.91–1.76 (m, 3H), 1.72–1.56 (m, 2H), 1.22 and 1.21 (2t, 6H, *J* 7.1). δ_C (d_6-DMSO) 142.06, 138.84, 128.72, 128.60, 128.55, 128.45, 128.37, 127.75, 126.21, 75.47, 61.29 (d, $^2J_{CP}$ 7.1), 49.48 (d, $^3J_{CP}$ 8.7), 33.12 (d, $^3J_{CP}$ 8.7), 32.62 (d, $^1J_{CP}$ 138.4), 30.52 (d, $^2J_{CP}$ 3.1), 25.89 (d, $^2J_{CP}$ 3.1), 16.70 (d, $^3J_{CP}$ 5.6).

General Procedure for the Preparation of [3-(Benzyloxyformylamino)-1-phenethylpropyl]phosphonic Acid Diethyl Ester ***7***

Formic acid (500 mmol) was treated with acetic acid anhydride (50 mmol), and the mixture stirred under exclusion of humidity. After 20 min, the solution was cooled to 0°C, and the hydroxylamine **6** (10 mmol), dissolved in dry THF (20 mL), was added dropwise. After 10 min, the mixture was allowed to warm to room temperature and stirred for another hour. The solution was treated with EtOAc (200 mL), and successively washed with water (3 × 50 mL), aqueous KOH (0.1 M, 3 × 25 mL), and once again with water. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc) to give compound **7** as a pale yellow oil (94%). Calc. for $C_{23}H_{32}NO_5P$ $[M+H]^+$: 434.2096. Found: 434.2120. ν_{max} (KBr)/cm^{-1} 1678 (C=O), 1233 (P=O). δ_H (d_6-DMSO) 8.29–7.90 (br s, 1H), 7.45–7.35 (m, 5H), 7.30–7.26 (m, 2H), 7.20–7.17 (m, 3H), 4.91 (s, 2H), 4.04–3.94 (m, 4H), 3.78–3.52 (m, 2H), 2.72–2.60 (m, 2H), 1.99–1.63 (m, 5H), 1.21 and 1.20 (2t, 6H, *J* 7.1). δ_C ($CDCl_3$) 162.98, 141.35, 134.33, 129.47, 129.11, 128.74, 128.47, 126.07, 77.68, 61.74 (d, $^2J_{CP}$ 6.6), 61.72 (d, $^2J_{CP}$ 7.1), 42.37, 33.43 (d, $^3J_{CP}$ 9.7), 32.96 (d, $^1J_{CP}$ 139.4), 30.25, 25.67, 16.52 (d, $^3J_{CP}$ 5.6).

General Procedure for the Preparation of [3-(Acetylbenzyloxyamino)-1-phenethylpropyl]phosphonic Acid Diethyl Ester ***8***

Acetic acid anhydride (20 mmol) was added to a solution of hydroxylamine **6** (10 mmol) in dry THF (10 mL), and the mixture stirred at room temperature for 2 h. After addition of EtOAc (100 mL), the organic layer was washed with aqueous KOH (0.1 M, 2 × 50 mL), water (50 mL), and three times with aqueous HCl (1 M). The organic layer was dried over $MgSO_4$, evaporated, and the residue was purified by column chromatography on silica gel (EtOAc) to give compound **8** as pale yellow oil (83%). Found: C 64.0, H 7.8, N 3.5. $C_{24}H_{34}NO_5P$ requires C 64.4, H 7.7, N 3.1%. ν_{max} (KBr)/cm^{-1} 1661 (C=O), 1233 (P=O). δ_H (d_6-DMSO) 7.45–7.35 (m, 5H), 7.30–7.26 (m, 2H), 7.20–7.16 (m, 3H), 4.88 (s, 2H), 4.04–3.94 (m, 4H), 3.81–3.63 (m, 2H), 2.72–2.59 (m, 2H), 2.02 (s, 3H), 1.99–1.63 (m, 5H), 1.21 and 1.20 (2t, 6H, *J* 7.1). δ_C ($CDCl_3$) 172.20, 141.47, 134.45, 129.25, 128.96, 128.70, 128.49, 128.42, 125.98, 76.30, 61.66 (d, $^2J_{CP}$ 7.1), 43.65, 33.42 (d, $^3J_{CP}$ 10.2), 33.05 (d, $^1J_{CP}$ 139.4), 30.22 (d, $^2J_{CP}$ 3.1), 25.55, 20.56, 16.52 (d, $^3J_{CP}$ 6.1), 16.49 (d, $^3J_{CP}$ 6.1).

General procedure for the Preparation of [3-(Benzyloxyformylamino)-1-phenethylpropyl]-(2,2-dimethylpropionyloxymethoxy)phosphinoyloxymethyl Ester ***9*** *and [3-(Benzyloxyacetylamino)-1-phenethylpropyl]-(2,2-dimethylpropionyloxymethoxy)phosphinoyloxymethyl Ester* ***10***

Trimethylsilyl bromide (15 mmol) was added to a stirred solution of phosphonic acid diethyl esters **7** or **8** (3 mmol) in dry CH_2Cl_2 (10 mL) at 0°C. After 1 h, the solution was allowed to warm to room temperature and stirred for 24 h. The solvent was removed under reduced pressure, and the residue was dissolved in THF (10 mL) and treated with water (0.1 mL). After 5 min, the solvent was evaporated and the residue was dried in vacuo overnight. For the alkylation step, the resulting oil was dissolved in anhydrous DMF (20 mL), treated with triethylamine (9 mmol), and stirred for 5 min. Chloromethyl pivalate (30 mmol) was added and the mixture was stirred at 70°C for 2 h. Another 3 mmol of triethylamine and 5 mmol of chloromethyl pivalate were added, and the solution was stirred for 2 h. The procedure of adding triethylamine and chloromethyl pivalate was repeated once again. After an overall time of 6 h, the mixture was allowed to cool and stirred overnight at room temperature. Diethyl ether (100 mL) was added, and the solution was successively washed with water (50 mL), saturated aqueous $NaHCO_3$ solution (2 × 50 mL), and again with water (50 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was purified by column chromatography on silica gel (Et_2O) to give compounds **9** and **10** as colourless products.

Compound **9** was obtained as a colourless oil (37%). Found: C 61.2, H 7.3, N 2.5. $C_{31}H_{44}NO_9P$ requires C 61.5, H 7.3,

N 2.3%. ν_{max} (KBr)/cm^{-1} 1753 (C=O, ester), 1680 (C=O, hydroxamate), 1255 (P=O). δ_H ($CDCl_3$) 8.27–7.88 (br s, 1H), 7.44–7.24 (m, 7H), 7.21–7.13 (m, 3H), 5.63 (ddd, 4H, $^3J_{HP}$ 12.5, J_{AB} 7.9), 4.90 (s, 2H), 3.74–3.53 (m, 2H), 2.68–2.58 (m, 2H), 1.99–1.60 (s, 5H), 1.14 and 1.13 (2s, 18H). δ_C ($CDCl_3$) 176.86, 162.99, 140.88, 134.34, 130.88, 129.54, 129.14, 128.81, 128.77, 128.51, 128.44, 126.18, 81.51, 77.65, 42.09, 38.73, 33.70 (d, $^1J_{CP}$ 138.4), 33.26 (d, $^3J_{CP}$ 9.7), 29.78, 26.86, 25.30.

Compound **10** was obtained as a colourless oil (70%). Found: C 61.7, H 7.5, N 2.2. $C_{32}H_{46}NO_9P$ requires C 62.0, H 7.5, N 2.3%. ν_{max} (KBr)/cm^{-1} 1753 (C=O, ester), 1666 (C=O, hydroxamate), 1252 (P=O). δ_H ($CDCl_3$) 7.45–7.37 (m, 5H), 7.29–7.26 (m, 2H), 7.20–7.14 (m, 3H), 5.62 (ddd, 4H, $^3J_{HP}$ 12.7, J_{AB} 7.1), 4.87 (s, 2H), 3.77–3.60 (m, 2H), 2.68–2.59 (m, 2H), 2.00 (s, 3H), 1.98–1.61 (m, 5H), 1.13 and 1.12 (2s, 18H). δ_C ($CDCl_3$) 176.82, 172.26, 140.99, 134.40, 129.31, 128.97, 128.71, 128.45, 126.07, 81.45 (d, $^2J_{CP}$ 7.1), 76.34, 43.42, 38.71, 33.70 (d, $^1J_{CP}$ 138.4), 33.20 (d, $^3J_{CP}$ 10.2), 29.75 (d, $^2J_{CP}$ 3.1), 26.84, 25.13 (d, $^2J_{CP}$ 2.0), 20.51.

*General Procedure for the Preparation of [3-(Formylhydroxyamino)-1-phenethylpropyl]-(2,2-dimethylpropionyloxymethoxy)phosphinoyloxymethyl Ester **11** and [3-(Formylhydroxyamino)-1-phenethylpropyl]-(2,2-dimethylpropionyloxymethoxy)phosphinoyloxymethyl Ester **12***

The *O*-protected hydroxamic acid **9** or **10** (10 mmol) was dissolved in methanol (50 mL). After addition of the Pd–C catalyst, hydrogen gas was added to generate a pressure of 2 bar, and the mixture was hydrogenated for 1 h. The suspension was filtered through an SPE tube RP-18 purchased from Supelco. The filtrate was evaporated to give compounds **11** and **12** as colourless oils.

Compound **11** was obtained as a colourless oil (99%). Found: C 56.0, H 7.7, N 2.9. $C_{24}H_{38}NO_9P$ requires C 55.9, H 7.4, N 2.7%. ν_{max} (KBr)/cm^{-1} 1754 (C=O, ester), 1673 (C=O, hydroxamate), 1251 (P=O). δ_H (d_6-DMSO) 10.04 (s, 0.5H), 9.60 (s, 0.5H), 8.24 (s, 0.5H), 7.88 (s, 0.5H), 7.31–7.25 (m, 2H), 7.21–7.15 (m, 3H), 5.63 (ddd, 2H, $^3J_{HP}$ 12.4, J_{AB} 8.1), 3.64–3.45 (m, 2H), 2.73–2.59 (m, 2H), 2.00–1.59 (m, 5H), 1.15 and 1.14 (2s, 18H). δ_C ($CDCl_3$) 176.91, 176.89, 163.53, 140.62 (d, $^3J_{CP}$ 10.2), 128.59, 128.42, 128.34, 126.30, 81.83 (d, $^2J_{CP}$ 7.1), 44.81 (d, $^3J_{CP}$ 4.1), 38.74, 34.00 (d, $^1J_{CP}$ 137.4), 33.55 (d, $^3J_{CP}$ 13.2), 30.78 (d, $^2J_{CP}$ 4.1), 26.84, 24.50 (d, $^2J_{CP}$ 3.1).

Compound **12** was obtained as a colourless oil (100%). Found: C 56.4, H 7.8, N 2.7. $C_{25}H_{40}NO_9P$ requires C 56.7, H 7.6, N 2.6%. ν_{max} (KBr)/cm^{-1} 1752 (C=O, ester), 1654 (C=O, hydroxamate), 1232 (P=O). δ_H (d_6-DMSO) 9.77 (s, 1H), 7.31–7.25 (m, 2H), 7.15 (m, 3H), 5.63 (ddd, $^3J_{HP}$ 12.7, J_{AB} 7.1), 3.68–3.47 (m, 2H), 2.71–2.59 (m, 2H), 1.99–1.62 (m, 8H), 1.15 and 1.14 (2s, 18H). δ_C ($CDCl_3$) 176.87, 176.82, 172.80, 140.69, 128.57, 128.35, 126.25, 81.77 (d, $^2J_{CP}$ 6.1), 81.49 (d, $^2J_{CP}$ 7.1), 45.88 (d, $^3J_{CP}$ 4.1), 38.76, 38.74, 34.04 (d, $^1J_{CP}$ 137.4), 33.55 (d, $^3J_{CP}$ 13.2), 30.84 (d, $^2J_{CP}$ 4.1), 26.85, 24.80 (d, $^2J_{CP}$ 2.0), 20.58.

Determination of in vitro Antimalarial Activity

Culture of P. falciparum

The *P. falciparum* 3D7 strain was maintained in continuous culture, according to Trager, Jensen, and Das Gupta et al.[12] The parasites were grown in human red blood cells (blood group A positive), RPMI 1640 medium supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, and 0.5% AlbuMAX (Invitrogen, Karlsruhe, Germany) at 5% hematocrit. The flasks were gassed with 90% N_2, 5% O_2, and 5% CO_2, and incubated at 37°C. The development of the cultures and the percentage of infected red blood cells were determined by light microscopy of Giemsa-stained thin smears.

Preparation of Drug Solutions

The respective compounds (20 μmol) were dissolved in DMSO (400 μL) and further diluted with water/ethanol (50:50) to obtain the particular concentration.

Determination of Parasite Growth Inhibition

The tests were carried out in 96-well microtitre plates under strict aseptic conditions, according to the literature. Dilutions of each compound were added to a suspension (250 μL) of *P. falciparum* infected erythrocytes (1.5% hematocrit, 1.5–2% parasitemia). The plates were flushed with a gas mixture consisting of 90% N_2, 5% O_2, and 5% CO_2, closed tightly and incubated at 37°C for 24 h. Afterwards, 0.1 μCi of 8-[^{3}H]-hypoxanthine was added to each well. The plates were flushed with the above-mentioned gas mixture, incubated for an additional 24 h at 37°C, and subsequently harvested with a cell harvester system (Inotech, Dottikon, Switzerland). Infected erythrocytes were washed four times with distilled water before they were analyzed for incorporated radioactivity in a multidetector liquid scintillation counter (Wallac, Turku, Finland).

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