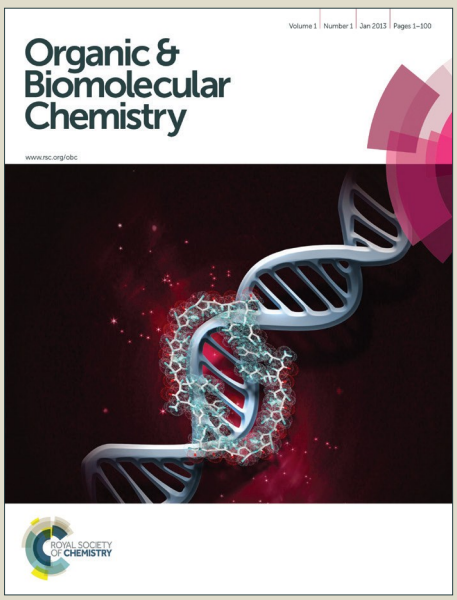
Organic & Biomolecular Chemistry

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Journal Name

ARTICLE

Facile Synthesis of the NNRTI Microbicide MC1220 and Synthesis of its Phosphoramidate Prodrugs

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

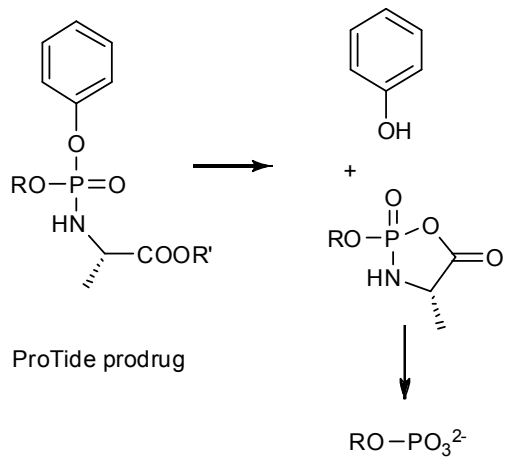
Yasser M. Loksha,[a,b] Erik B. Pedersen,[a]* Paolo La Colla,[c] Roberta Loddo[c]

A facile and novel synthetic route of MC-1220 was achieved by condensation of 4,6-dichloro-*N,N*,5-trimethylpyrimidin-2-amine (**1**) with the sodium salt of 2,6-difluorophenylacetonitrile, followed by methylation and strong acidic hydrolysis. The prodrugs of MC-1220 were synthesized by reaction of chlorophosphoramidate derivatives (**7a-e**) or α-acetobromoglucose with the sodium salt of MC-1220. The stability and anti-HIV-1 activity of phosphoramidate prodrugs turned out comparable to those of the parent drug MC-1220.

Introduction

It is believed that more than 40% NCEs (new chemical entities) are too insoluble in water. A solution for that is one of the most challenging aspects of drug development process.[1] Furthermore, it has been reported that phenolic drugs may have various undesirable drug properties that may become pharmacological, pharmaceutical or pharmacokinetic barriers for application. To overcome poor physicochemical properties of phenolic drugs, their corresponding ester, sulphate, carbamate, carbonate, phosphate and ether prodrugs have been investigated.[2]

McGuigan and colaborators have developed a technology, known as the phosphoramidate Pro Tide method[3,4] to mask the phosphate group charges in order to provide efficient passive cell-membrane penetration (Scheme **1**). The typical Pro Tide prodrug is a phenyl phosphoramidate prepared from the ester of an amino acid. Upon entering the cell, the masking groups are enzymatically cleaved to release the phosphorylated biomolecule. The initial step to release the drug is the ester hydrolysis, which causes the intracellular displacement of the phenoxy group by the carboxylate anion.[5] Although not attempted yet, it is obvious that this route also could be suitable to deliver phenolic componds into the cells. Furthermore, we hypothesized that one could go one step further to heteroaromatic oxo compounds which could be released from the prodrug in their corresponding enol form. We have previously reported MC-1220 to induce partial protection against human immunodeficiency virus (HIV) after intravaginal challenge of rhesus macaques.[6-8] MC-1220 has been claimed to possess some potential for treatments to be started immediately after an HIV-1 infection is believed to have occurred.[9]



Scheme 1. Proposed activation pathway of a Pro Tide prodrug.

Following the recognition of human immunodeficiency virus (HIV) as the aetiological agent of the acquired immunodeficiency syndrome (AIDS), a great deal of research activities was directed to provide effective treatments. Among the compounds demonstrating potent and selective anti-HIV-1 activity emerged the class of non-nucleoside reverse transcriptase inhibitors (NNRTIs).[10-17] Some of these showed potential as microbicides to prevent sexual HIV-1 transmission/acquisition.[6-9,18,19] Having experienced the need of liposome formulation of MC-1220,[6,8] we therefore chose this compound in order to get insight into the potential of our hypothesis of using the Pro Tide type approach to make a prodrug for a heterocyclic compound by introducing it as a

[a] *Nucleic Acid Center†, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark.*
[b] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Industries, Sinai University, North Sinai, Al-Arish, Egypt.*
[c] *Department of Biomedical Sciences, Section of General Microbiology and Virology and Microbial Biotechnologies, University of Cagliari, 09042 Monserrato, Italy.*

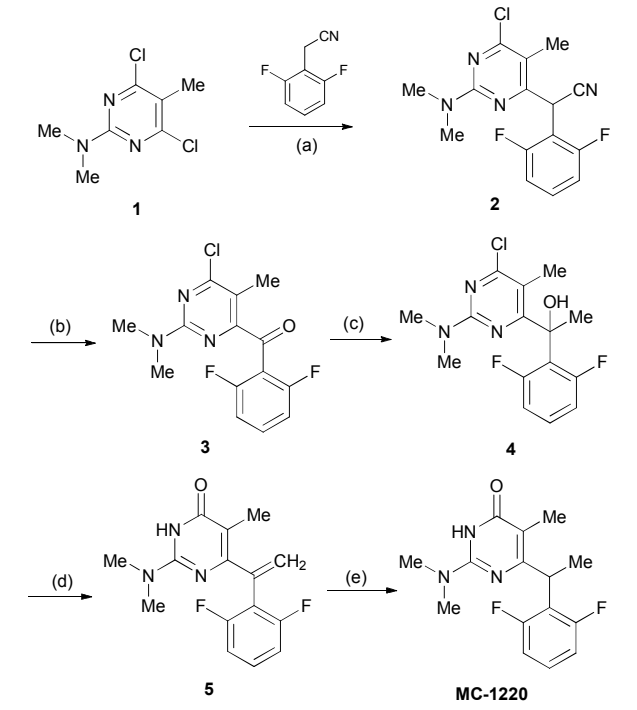
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

phenolic part of a Pro Tide type prodrug. It was hypothesized that the heterocyclic moiety would be the better leaving group in the prodrug because of its electron attracting nitrogen atoms.

Results and Discussion

Synthesis of MC-1220

The synthesis of MC-1220 and its analogues has been previously carried out by Mai et al.[20] and Bartolini et al.[21] by condensation of the corresponding β-keto esters with guanidine sulfate derivatives. The β-keto esters, however, have been prepared through multistep reactions.[20] Recently, Radi *et al*. have reported the synthesis of arylmethyl-functionalized S-DABOs and related analogues from C6 protected formyl pyrimidinone.[22] Then, we have synthesized MC-1220 by nucleophilic substitution on previously reported[23,24] 4,6-dichloro-*N,N*,5-trimethylpyrimidin-2-amine (**1**) with the sodium salt of 2,4-difluorophenylacetonitrile to afford 2-[6-chloro-2-(dimethylamino)-5-methyl-pyrimidin-4-yl]-2-(2,6-difluorophenyl)acetonitrile (**2**). A stream of oxygen was bubbled through the reaction mixture of compound 2, and sodium hydride in DMF for 3 hours to afford 4-(2,6-difluorobenzoyl)-6-chloro-*N,N*,5-trimethylpyrimidin-2-amine (**3**). Reaction of methylmagnesium bromide with compound **3** afforded the tertiary alcohol **4**, which has been treated with a strong acidic medium to afford 6-[1-(2,6-difluorophenyl)vinyl]-2-(dimethylamino)-5-methylpyrimidin-4(3*H*)-one (**5**).

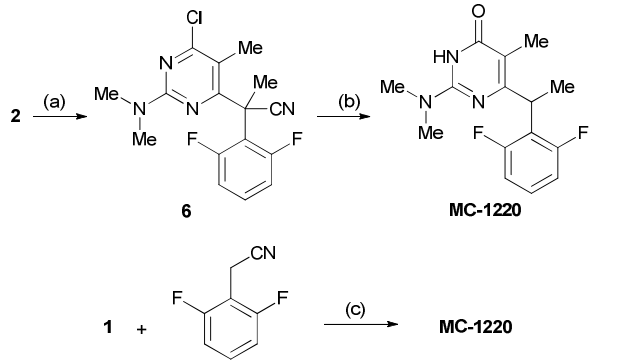


Scheme 2. Our previous synthesis of MC-1220. **Reagents and conditions:** (a) NaH, DMF, RT; (b) i) NaH, DMF, RT, ii) O_2; (c) i) CH_3MgBr, Et_2O, ii) H_2O, H^+; (d) 6M HCl, reflux 90 h; (e) 10% Pd/C, EtOH, 3.5 bar H_2.

Reduction of the vinylic group of compound **5** furnished the desired compound MC-1220 (Scheme **2**).[24] To reduce the number of steps for the synthesis of MC-1220 in high overall yield, we now report a one-pot synthesis of MC-1220 in 74 % yield.

Compound **2** was synthesized from **1** in 97% yield, as previously described.[24] Successful methylation of compound **2** was achieved by addition of methyl iodide to a mixture of compound **2** and sodium hydride in *N,N*-dimethylformamide at room temperature under dry conditions to afford 2-[6-chloro-2-(dimethylamino)-5-methylpyrimidin-4-yl]-2-(2,6-difluorophenyl)propanenitrile (**6**) in 86% yield. MC-1220 was obtained in 97% yield by the hydrolysis of compound **6** in 8 M hydrochloric acid for 90 hours. The stepwise synthesis of MC-1220 from compound **1** was carried out with an overall yield of 81% (Scheme **2**).

In the attempt to synthesize MC-1220 in a one pot reaction without separating the intermediates **2** and **6**, sodium hydride was added to a solution of compound **1** and 2,6-difluorophenylacetonitrile, followed by addition of methyl iodide after stirring for 0.5 hour. The product in the mixture was hydrolyzed by addition of concentrated hydrochloric acid and refluxing for 90 hours. MC-1220 was precipitated in 74% yield by neutralization of the cold concentrated reaction mixture with 10% sodium hydroxide (Scheme **3**).



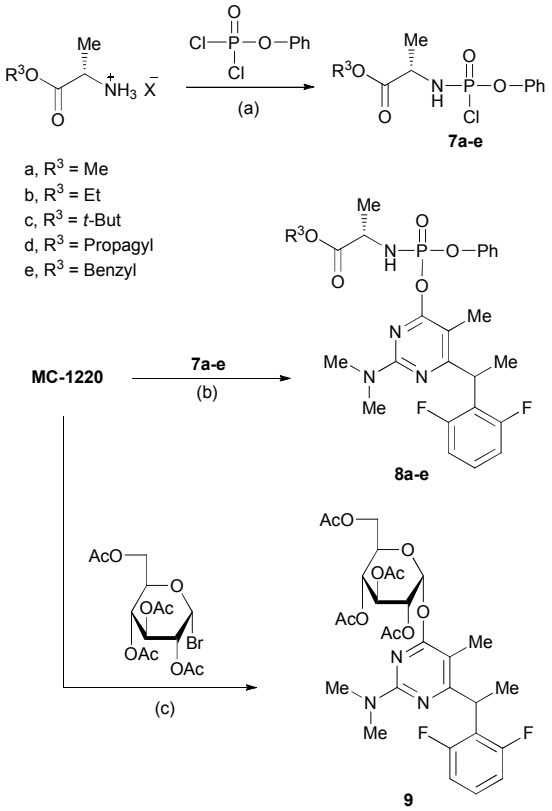
Scheme 3. Facile synthesis of MC-1220. **Reagents and conditions:** (a) CH_3I, NaH, DMF, RT; (b) 8M HCl, reflux 90 h; (c) i) NaH, DMF, RT, ii) CH_3I, RT, iii) 8M HCl, reflux 90 h.

Prodrugs of MC-1220

(2*S*)-2-[Chloro(phenoxy)phosphorylamino]propanoate esters (**7a-e**) were synthesized by treatment of L-alanine ester hydrochlorides with phenyl phosphorodichloridate at -80 ^{o}C in the presence of triethylamine, as previously described for **7a-c,e** by McGuigan and Balzarini.[25] The prodrugs of MC-1220 (**8a-e**) were obtained by reaction of chlorophosphoramidates **7a-e** with the sodium salt of MC-1220 in *N,N*-dimethylformamide (Scheme **4**).

Assuming easy hydrolysis of the glycoside bond in serum, we thought it could be possible also to use an O-glucosylated derivative of MC-1220 as a prodrug. Such a compound was synthesized by reaction of MC-1220 with (2*R*,3*R*,4*S*,5*R*,6*R*)-2-(acetoxymethyl)-6-bromotetrahydro-2*H*-pyran-3,4,5-triyl triacetate to afford the α-anomeric product **9**. Because MC-

1220 is used in its racemic form, the prodrug **9** is obtained as a mixture of two α-anomers. One of them was isolated as a nearly pure diastereomer by repeated chromatography. The assignment of the anomeric configuration was based on comparison of ^{1}H and ^{13}C NMR chemical shifts with those of the corresponding acetylated phenyl glucoside.[26,27]



Scheme 4. Synthesis of MC-1220 prodrugs. **Reagents and conditions:** (a) Et_3N, -80 °C, CH_2Cl_2; (b) NaH, DMF, 5 min., RT; (c) NaH, DMF, 30 min., RT

Anti-HIV-1 assays

MT-4 cell-based assays (Table **1**) were used to evaluate the cytotoxicity of MC-1220 and of its newly synthesized prodrugs, as well as their activity against the wild type HIV-1_{IIIB} strain and a number of NNRTI-resistant mutants carrying different clinically significant mutations. The phoshoramidate prodrugs **8a-e** proved slightly more cytotoxic (25-40 μM) than the parent compound (>100 μM). They also proved as potent as MC-1220 against both the wild type and the Y181C resistant mutant. Moreover, like MC-1220 they proved inactive against the resistant variants bearing the 103 + 181 mutation. Noteworthy, the propargyl derivative **8d** proved three-folds more potent than MC-1220 against HIV-1 wild type and two-folds more potent against the 181 mutant. *Vice versa*, compound **9** showed a hundred-fold lower potency against HIV-1 wild type and no activity at all against the NNRTI-resistant mutants.

Stability of MC-1220 prodrugs in aqueous media

Due to the fact that, in cell-based assays, both cytotoxicity and antiviral activity are determined after 96 hours incubation at 37 °C, the stability of test MC-1220 prodrugs in RPMI containing 10% foetal calf serum (FCS), at 37 °C, was checked by HPLC in samples withdrawn from time to time until 100% hydrolysis, or until the end of incubation under the above cell-free conditions. As an example, the hydrolysis kinetics of prodrug **8a** are shown in Figure **1**. It can be seen that, within 76 hours of incubation, 8a is completely hydrolyzed to MC-1220 (peak c), thus progressively disappearing as prodrug (peak d).

Compounds **8a,b** and **d** were hydrolyzed to MC-1220 by 100%, 32% and 51%, respectively, after a 96 hours incubation in RPMI medium addition of 10% FCS (see last column of Table **1**). Viceversa, under the same experimental conditions compounds **8c** and **e** underwent only 5% hydrolysis. Since, in cell-based assays, all prodrugs show high antiviral potency, we assume that they are easily enzymatically hydrolyzed after their uptake into the cells as described for Pro Tide prodrugs.[5] This is particularly true for compounds **8c** and **e** which, after a 96 hours incubation in cell-free RPMI are hydrolyzed to MC-1220 by only 5%. On the other hand, no hydrolysis to MC-1220

Table 1. Cytotoxicity and anti-HIV-1 activity of **MC-1220**, its prodrugs **8a-e, 9** and MKC-442 and Efavirenz (EFV) as reference compounds.

Compd.	R	CC_{50} (μM)[b]	EC_{50} (μM)[a]				SI[d]	% Hydrol.[e]
			Wild type	EFV[R c]	Y181C	K103N + Y181C		
8a	Me	25±3	0.007±0.002	>25	1.2±0.2	>25	3571	100
8b	Et	40±1	0.006±0.001	15±4	1±0.05	>40	6666	32
8c	*t*-But	36±0.5	0.008±0.001	>36	1±0.3	>36	4500	5
8d	CH≡C-CH_2	40±1	0.003±0.0001	15±3	0.5±0.05	>40	13333	51
8e	Benzyl	36±0.5	0.01±0.002	18±2	1.1±0.1	>36	3600	5
9		>100	1	>100	>100	>100	>100	0
MC-1220		>100	0.01±0.003	>100	0.7±0.2	90±10	>10000	
MKC-442		>100	0.03	100	20	>100	>3333	
EFV		30	0.002	3	0.008	0.3	15000	

[a]Compound dose required to achieve 50 % protection of MT-4 cells from HIV-1-induced cytopathogenicity, as determined by the MTT method.
[b]Compound dose required to reduce the viability of mock-infected cells by 50 %, as determined by the MTT method.
[c]The mutations are K100R, V179D and P225H.
[d]Selectivity index: ratio CC_{50}/EC_{50}.
Data are mean values of at least two separate experiments. The symbol (>) indicates that cytotoxicity/antiviral activity was not seen at the indicated concentration.
[e]Percent hydrolysis of the MC-1220 prodrugs in culture medium (RPMI) after a 4 day incubation, as determined by HPLC disintegration chromatograms.

was detected with the glucoside prodrug **9** in cell-free RPMI even after 96 hour incubation (Table **1**), which explains its inactivity against HIV-1 in cell-based assays.

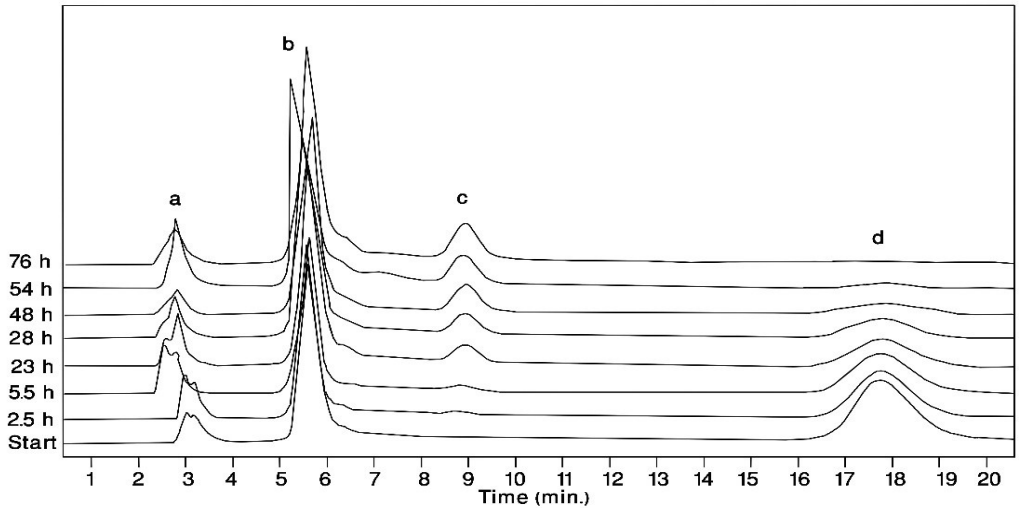


Figure 1. HPLC chromatograms showing the hydrolysis of the prodrug **8a**: i) peaks a (RPMI) and b (FCS) were of similar magnitude in all chromatograms; ii) The progress in hydrolysis of **8a** (peak d) into MC1220 (peak c) could be followed by using the peaks a and b as internal references.

Experimental

Chemistry

NMR spectra were recorded on a Varian Gemini 2000 NMR spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C with TMS as internal standard. MALDI mass spectra were recorded on a 4.7 Tesla Ultima (IonSpec, Irvine, CA) Fourier Transform Ion Cyclotron Resonance (FTICR) Mass Spectrometer. Melting points were determined on a Büchi melting point apparatus. Elemental analyses were performed at H.C. Ørsted Institute, University of Copenhagen. Silica gel (0.040–0.063 mm) used for column chromatography and analytical silica gel TLC plates 60 F_{254} were purchased from Merck. Solvents for chromatography were bought as HPLC grade or distilled prior to use. Reactions were in general carried out under argon atmosphere. CH_3CN was dried over 3 Å molecular sieves.

2-[6-Chloro-2-(dimethylamino)-5-methylpyrimidin-4-yl]-2-(2,6-difluorophenyl)propanenitrile (6).

Sodium hydride (0.6 g, 55% suspension in paraffin oil, 14 mmol) was added portionwise to a solution of compound **2** (3.22 g, 10 mmol) in dry *N*,*N*-dimethylformamide (15 mL) with stirring for 15 min at room temperature. Methyl iodide (0.76 mL, 12 mmol) was added in one portion to the reaction mixture and stirred for 1.5 h. The reaction was quenched with ethanol (1 mL) followed by addition of cold water (50 mL) and then ether (60 mL) was added. The mixture was shacken and the two layers were separated. The ether layer was dried over magnesium sulfate and the solvent was evaporated under reduced pressure. The residual material was dried by vacuum to afford 2.9 g of compound **6**. Yield 86.3% as an oil; ^{1}H NMR ($CDCl_3$) δ: 1.97 (s, 3H, CH_3-C-CN), 2.17 (s, 3H, CH_3-C5), 3.19 [s, 6H, $(CH_3)_2N$], 6.91 (t, 2H, *J* = 8.7 Hz, H_{arom}), 7.27–7.33 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 13.97 (*C*H_3-C5), 25.70 (*C*H_3-C-CN), 36.89 [$(CH_3)_2N$], 112.27 (C5), 112.49-112.83 (m, C_{arom}), 119.92 (CN), 130.31 (t, J_{CF} = 10.9 Hz, C_{arom}), 159.87 (dd, J_{CF} = 6.4, 252.5 Hz, C_{arom}), 159.18 (C4), 163.05 (C2), 164.43 (C6); HRMS (MALDI): *m/z* calcd. for $C_{16}H_{16}ClF_2N_4$ (MH^+) 337.1026, found 337.1037.

6-[1-(2,6-Difluorophenyl)ethyl]-2-(dimethylamino)-5-methylpyrimidin-4(3*H*)-one (MC-1220).

Compound **6** (0.01 mol, 3.36 g) was refluxed in a mixture of concentrated hydrochloric acid (20 mL), acetic acid (10 mL) and water (10 mL) at 115°C for 40 hours. The solvent was evaporated under reduced pressure and the residue was treated with water (20 mL) and neutralized with 10% aqueous sodium hydroxide. The crystallized product in the solution was filtered off and dried to afford 2.85 g of MC-1220. Yield 97%; as a white solid; mp 166–168 °C, lit.[24] mp 166-168 °C.

One pot synthesis of MC-1220.

To a solution of compound **1** (8.24, 40.0 mmol) and 2,6-diflourophenylacetonitrile (6.5 g, 42.5 mmol) in dry *N*,*N*-dimethylformamide (75 mL) under dry conditions (using $CaCl_2$ tube),was added sodium hydride (5.3 g of 55% suspension in paraffin oil, 120.0 mmol) portionwise at room temperature. After complete addition, the reaction mixture was stirred at room temperature for 0.5 hour. Methyl iodide (3 mL, 48.0 mmol) was added dropwise to the reaction mixture and stirred for 2 hours. Acetic acid (5 mL was added dropwise) followed by addition of concentrated hydrochloric acid (200 mL, 37%) and the mixture was refluxed for 90 hours. The solution was cooled to room temperature and filtered and the mixture was concentrated to 10 mL under reduced pressure. Water (100

mL) was added and the suspension was neutralized with 10% aqueous sodium hydroxide. The precipitate was filtered off and dried to give 8.7 g of the pure MC-1220; yield 74%; as a white solid; mp = 166–168 °C, lit.[24] mp = 166-168 °C.

Synthesis of (2*S*)-2-[chloro(phenoxy)phosphorylamino]propanoates (7a-e). Under nitrogen, a solution of triethylamine (1.4 mL, 10 mmol) in anhydrous methylene chloride (5 mL) was added dropwise at –78°C to a solution of phenyl phosphorodichloridate (1.12 g, 5 mmol) and the hydrochloride of L-alanine ester derivatives (5 mmol) in anhydrous methylene chloride (15 mL) with stirring. After complete addition, the mixture was allowed to reach room temperature gradually and left to be stirred overnight. The solvent was removed under reduced pressure and the residual material was treated with ether (20 mL) and filtered. The filtrate was collected and evaporated under reduced pressure to give compounds **7a-e** as a crude oil. The product is unstable, so it should be stored under nitrogen or in a vial evacuated from air by nitrogen. The product was pure enough to be used for the next step.
Yield: **7a** (R = Me) 56%,[25] **7b** (R = Et) 91%,[25], **7c** (R = *t*-Bu), 88%,[25] **7e** (R =Benzyl) 91%.[25]

(2*S*)-Prop-2-ynyl 2-[chloro(phenoxy)phosphorylamino]-propanoate (7d). Yield 90%; as an oil; ^{1}H NMR ($CDCl_3$) δ: 1.52–1.56 (m, 3H, C*H*$_3$CH), 2.50–2.54 (m, 1H, HC≡C), 4.23 (bs, 1H, CH$_3$C*H*), 4.49 (bs, 1H, NH), 4.75–4.79 (m, 2H, CH_2), 7.24–7.42 (m, 5H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 20.22, 20.35 (*C*H$_3$CH), 50.33, 50.57 (CH$_3$*C*H), 53.12, 53.20 (CH_2), 75.67, 75.73 (*C*H≡C), 80.16, 80.72 (CH≡*C*), 120.44, 120.52, 125.98, 126.00, 127.00, 129.87, 129.89, 130.18 (C_{arom}), 171.88 (COO); ^{15}P NMR ($CDCl_3$) δ: 8.74, 8.97; HRMS (MALDI): *m/z* calcd. for $C_{12}H_{13}ClNO_4P$ (M^+) 301.0265, found 301.0262.

Synthesis of MC-1220 prodrugs (8a-e).

Under dry conditions (using $CaCl_2$ tube), sodium hydride (0.65 g, 55% suspension in paraffin oil, 1.5 mmol) was added portionwise to a solution of MC-1220 (293 mg, 1 mmol) in dry *N*,*N*-dimethylformamide (5 mL) with stirring at 0 °C. Compound **7a-e** (1.5 mmol) was added to the reaction mixture in one portion, the stirring was continued for 0.5h at room temprature. Ether (15 mL) was added followed by addition of water (15 mL) to the mixture and stirred for 5 min. The two layers were separated and the water layer was extracted two times with ether (2 x 10 mL) and the combined ether phases were dried and evaporated under reduced pressure. The residual material was purified by a column chromatography on silica gel using ether as an eluent to afford the non-separable diastereomers of compounds **8a-e** as oils.

(2*S*)-Methyl 2-{[6-(1-(2,6-difluorophenyl)ethyl)-2-(dimethylamino)-5-methylpyrimidin-4-yloxy](phenoxy)-phosphorylamino}propanoate (8a). Yield: 56%; obtained as an oil of diastereomers; ^{1}H NMR ($CDCl_3$) δ: 1.34–1.46 (m, 3H, C*H*$_3$CH), 1.64 (d, 3H, *J* = 6.9 Hz, C*H*$_3$CH), 1.78, 1.83 (2s, 3H, CH$_3$-C5), 3.14 [s, 6H, $(CH_3)_2$N], 3.66, 3.69 (2s, 3H, OCH_3), 4.22–4.37 (m, 1H, CH$_3$C*H*), 6.78–6.84 (m, 2H, H_{arom}), 4.70–4.84 (m, 1H, NH), 7.07–7.35 (m, 6H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 9.07, 9.10 (CH$_3$-C5), 17.63 (*C*H$_3$CH), 21.49, 21.54 (*C*H$_3$CH-NH), 34.33 (CH$_3$*C*H), 36.92 [$(CH_3)_2$N], 50.31, 50.35 (CH$_3$*C*HNH), 52.36 (OCH_3), 102.09, 102.15, 102.22, 102.27 (C5), 111.21-111.55 (m, C_{arom}), 120.35–120.56 (m, C_{arom}), 124.92, 125.00 (C_{arom}), 127.85–128.16 (m, C_{arom}), 129.49, 129.55 (C_{arom}), 150.51, 150.86 (C_{arom}), 151.48 (C2), 159.20 (C4), 161.47 (dd, J_{CF} = 8.9, 248.4 Hz, C_{arom}), 171.38, 171.49 (C6), 173.63, 173.71 (COO); ^{15}P NMR ($CDCl_3$) δ: –1.23, –1.16, –1.10, –0.96; HRMS (MALDI): *m/z* calcd. for $C_{25}H_{30}F_2N_4O_5P$ (MH^+) 535.1916, found 535.1897.

(2*S*)-Ethyl 2-((6-(1-(2,6-difluorophenyl)ethyl)-2-(dimethyl-amino)-5-methylpyrimidin-4-yloxy)(phenoxy)phosphoryl-amino)propanoate (8b). Yield 64%; obtained as an oil of diastereomers; ^{1}H NMR ($CDCl_3$) δ: 1.21 (t, 3H, *J* = 7.2 Hz, C*H*$_3$CH$_2$), 1.34–1.45 (m, 3H, C*H*$_3$CH), 1.64 (d, 3H, *J* = 6.9 Hz, CH$_3$C*H*), 1.78, 1.83 (2s, 3H, CH$_3$-C5), 3.14 [s, 6H, $(CH_3)_2$N], 4.11 (q, 2H, *J* = 7.2 Hz, CH$_3$C*H*$_2$), 4.20–4.35 (m, 1H, CH$_3$C*H*), 4.56 (q, 1H, *J* = 6.9 Hz, CH$_3$C*H*), 4.71–4.85 (m, 1H, NH), 6.79–6.84 (m, 2H, H_{arom}), 7.06–7.32 (m, 6H, H_{arom}) ; ^{13}C NMR ($CDCl_3$) δ: 9.06, 9.09 (*C*H$_3$C5), 13.95, 14.01 (*C*H$_3$CH$_2$), 17.63 (*C*H$_3$CH), 21.46, 21.56 (*C*H$_3$CHNH), 34.31 (CH$_3$*C*H), 36.92 [$(CH_3)_2$N], 50.38, 50.41 (CH$_3$*C*NH), 61.35, 61.43 (CH_2O), 102.17, 102.22, 102.31, 102.33 (C5), 111.18-111.52 (m, C_{arom}), 120.16–120.54 (m, C_{arom}), 124.87, 124.96 (C_{arom}), 127.96 (t, J_{CF} = 10.6 Hz, C_{arom}), 129.45, 129.51, 150.49, 150.59 (C_{arom}), 152.84 (C2), 159.18 (C4), 161.45 (dd, J_{CF} = 8.7, 248.5 Hz, C_{arom}), 171.31, 171.40 (C6), 173.31, 173.44 (COO); ^{15}P NMR ($CDCl_3$) δ: –1.17, –1.09, –1.01, –0.88; HRMS (MALDI): *m/z* calcd. for $C_{26}H_{31}F_2N_4NaO_5P$ (MNa^+) 571.1892, found 571.1897.

(2*S*)-*tert*-Butyl 2-{[6-(1-(2,6-difluorophenyl)ethyl)-2-(dimethylamino)-5-methylpyrimidin-4-yloxy](phenoxy)-phosphorylamino}propanoate (8c). Yield 54%; obtained as an oil of diastereomers; ^{1}H NMR ($CDCl_3$) δ: 1.30–1.43 [m, 12H, $(CH_3)_3$C and C*H*$_3$CH), 1.64 (d, 3H, *J* = 6.9 Hz, C*H*$_3$CH), 1.78, 1.83 (2s, 3H, CH$_3$-C5), 3.14 [s, 6H, $(CH_3)_2$N], 4.07–4.25 (m, 1H, CH$_3$C*H*), 4.55 (q, 1H, *J* = 6.9 Hz, CH$_3$C*H*), 4.62–4.73 (m, 1H, NH), 6.77–6.84 (m, 2H, H_{arom}), 7.05–7.32 (m, 6H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 9.07, 9.09 (*C*H$_3$-C5), 17.64 (*C*H$_3$CH), 21.53, 21.58 (*C*H$_3$CH), 27.83 [(*C*H$_3$)$_3$C], 34.30 (CH$_3$*C*H), 36.91 [$(CH_3)_2$N], 50.89, 50.90 (CH$_3$*C*H-NH), 81.67, 81.74 [(CH$_3$)$_3$*C*], 111.17-111.52 (m, C_{arom}), 120.35–120.57 (m, C_{arom}), 124.82, 124.90 (C_{arom}), 127.92 (t, J_{CF} = 10.2 Hz, C_{arom}), 129.45, 129.50, 150.56, 150.65 (C_{arom}), 153.31 (C2), 159.23 (C4), 161.46 (dd, J_{CF} = 8.8, 248.3 Hz, C_{arom}), 171.24, 171.32 (C6), 172.41, 172.56 (COO); ^{15}P NMR ($CDCl_3$) δ: –1.08, –0.98, –0.89, –0.78; HRMS (MALDI): *m/z* calcd. for $C_{28}H_{35}F_2N_4NaO_5P$ (MNa^+) 599.2205, found 599.2180.

(2*S*)-Prop-2-ynyl 2-{[6-(1-(2,6-difluorophenyl)ethyl)-2-(dimethylamino)-5-methylpyrimidin-4-yloxy](phenoxy)-phosphorylamino}propanoate (8d). Yield 54%; obtained as an oil of diastereomers; ^{1}H NMR ($CDCl_3$) δ: 1.37–1.48 (m, 3H, C*H*$_3$CH), 1.64 (d, 3H, *J* = 7.2 Hz, C*H*$_3$CH), 1.78, 1.83 (2s, CH$_3$-C5), 2.17 (s, 1H, CH≡C), 3.14 [s, 6H, $(CH_3)_2$N], 4.28–4.40 (m, 1H,

CH_3CH), 4.54 (q, 1H, *J* = 7.2 Hz, CH_3CH), 4.61–4.78 (m, 3H, NH and CH_2), 6.78–6.86 (m, 2H, H_{arom}), 7.06–7.32 (m, 6H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 9.05, 9.09 (CH_3C5), 17.60 (CH_3CH), 21.24, 21.38 (CH_3CH-NH), 34.31 (CH_3CH), 36.93 [$(CH_3)_2N$], 50.26, 50.30 (CH_3CH-NH), 52.68, 52.74 (OCH_2), 75.32 (CH≡C), 76.90 (CH≡C), 102.13, 102.19, 102.25, 102.31 (C5), 111.20-111.55 (m, C_{arom}), 120.32–120.51 (m, C_{arom}), 124.93, 125.02 (C_{arom}), 127.97 (t, J_{CF} = 10.7 Hz, C_{arom}), 129.48, 129.54, 150.48, 150.53 (C_{arom}), 156.12 (C2), 159.16 (C4), 161.44 (dd, J_{CF} = 8.7, 248.4 Hz, C_{arom}), 171.36, 171.48 (C6), 172.49, 172.81 (COO); ^{15}P NMR ($CDCl_3$) δ: –1.42, –1.34, –1.28, –1.16; HRMS (MALDI): *m/z* calcd. for $C_{27}H_{30}F_2N_4O_5P$ (MH^+) 559.1916, found 559.1913.

(2*S*)-Benzyl 2-{[6-(1-(2,6-difluorophenyl)ethyl)-2-(dimethylamino)-5-methylpyrimidin-4-yloxy](phenoxy)-phosphorylamino}propanoate (8e). Yield 55%; obtained as oil of diasteeomers; ^1H NMR ($CDCl_3$) δ: 1.34–1.47 (m, 3H, CH_3CH), 1.64 (d, 3H, *J* = 6.9 Hz, CH_3CH), 1.76, 1.77, 1.81, 1.82 (4s, 3H, CH_3-C5), 3.09, 3.10 [2s, 6H, $(CH_3)_2N$], 4.27–4.43 (m, 1H, CH_3CH), 4.55 (q, 1H, *J* = 6.9 Hz, CH_3CH), 4.69–4.84 (m, 1H, NH), 5.04, 5.08 (2s, 2H, OCH_2), 6.77–6.85 (m, 2H, H_{arom}), 7.05–7.36 (m, 11H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 9.05, 9.09 (CH_3-C5), 17.61, 17.61 (CH_3CH), 21.45, 21.54 (CH_3CH), 34.31 (CH_3CH), 36.87 [$(CH_3)_2N$], 50.45 (CH_3CH-NH), 67.02, 67.10 (OCH_2), 102.15, 102.23, 102.27, 102.34 (C5), 111.19-111.54 (m, C_{arom}), 120.32–120.54 (m, C_{arom}), 124.88, 124.98, 127.84, 128.03, 128.35, 128.52, 128.54, 128.55, 135.20, 135.25, 150.40, 150.54 (C_{arom}), 154.24 (C2), 159.14 (C4), 161.45 (dd, J_{CF} = 8.6, 248.4 Hz, C_{arom}), 171.35, 171.43 (C6), 173.11, 173.28 (COO); ^{15}P NMR ($CDCl_3$) δ: –1.26, –1.15, –1.10, –0.96; HRMS (MALDI): *m/z* calcd. for $C_{31}H_{33}F_2N_4NaO_5P$ (MNa^+) 633.2049, found 633.2020.

(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(Acetoxymethyl)-6-{6-[1-(2,6-difluoro-phenyl)ethyl]-2-(dimethylamino)-5-methylpyrimidin-4-yl-oxy}tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (9). Sodium hydride (1.1 g of 55% suspension in paraffin oil, 24.6 mmol) was added portionwise to a solution of MC-1220 (6 g, 20.5 mmol) in dry *N,N*-dimethylformamide (50 mL), and mixture was stirred for 1 hour. The sodium salt of MC-1220 was added dropwise at 0ºC to a solution of α-acetobromoglucose (9.3 g, 22.6 mmol) in dry *N,N*-dimethylformamide (50 mL). After complete addition, the reaction mixture was left to reach room temperature with stirring for 2 hours. The reaction mixture was poured on ice-cold water (500 mL), ether (100 mL) was added and the two layers were separated. The water layer was extracted two times with ether (2 x 50) and the combined ether extracts were dried and evaporated under reduced pressure. The residue was chromatographed using a column of silica gel (ether/petroleum ether, 2:1, v/v) to give 6.1 g of **9** as oil. Yield 48% as mixture of two α-anomers; one of the α-anomers was purified by repeated chromatography; ^1H NMR ($CDCl_3$) δ: 1.63 (d, 3H, *J* = 7.2 Hz, CH_3CH), 1.87(s, 3H, CH_3-C5), 1.95, 2.02, 2.04, 2.05, (4 s, 12 H, CH_3CO), 3.07 [s, 6H, $(CH_3)_2N$], 3.86–3.91 (m, 1H, H5'), 4.14 (dd, 1H, J= 12.3, 2.4 Hz, H6'), 4.25 (dd, 1H, J= 12.3, 5.1 Hz, H6'), 4.58 (q, 1H, *J* = 7.1 Hz, CH-CH_3), 5.16 (t, 1H, *J* = 9.6 Hz, H4'), 5.27–5.37 (m, 2H, H2', H3'), 6.04 (d, 1H, *J* = 8.1 Hz, H1'), 6.74 – 6.84 (m, 2H, H_{arom}), 7.07 – 7.17 (m, 1H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ: 8.67 (CH_3-C5), 17.75 (CH_3CH), 20.55, 20.57, 20.60, 20.67 (Ac), 34.04 (CH_3CH), 36.54 ($[(CH_3)_2N]$, 61.98 (C6') 68.48, 70.39, 72.42, 72.95 (C2', C3', C4', C5'), 93.43 (C1'), 100.89 (C5), 111..08-111.43 (m, C_{arom}), 127.72 (t, J_{CF} = 10.7 Hz, C_{arom}), 159.45 (C2), 161.45 (dd, J_{CF} = 9.0, 247.8 Hz, C_{arom}), 165.58 (C4), 169.83 (C6), 169.24, 169.41, 170.24, 170.63 (COO); HRMS (MALDI): *m/z* calcd. for $C_{29}H_{35}F_2N_3NaO_{10}$ (MNa^+) 646.2183, found 646.2191.

Anti-HIV

Cell-based assays

Compounds

Compounds were dissolved in DMSO at 100 µM and then diluted in culture medium.

Cells and Viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. The cells supporting the multiplication of HIV-1 were the $CD4^+$ human T-cells (MT-4) containing an integrated HTLV-1 genome.

Cytotoxicity Assays

For cytotoxicity evaluations, exponentially growing MT-4 cells were seeded at an initial density of $1x10^5$ cells/ml in 96 well plates, with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G and 100 µg/mL streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 hrs at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.[28]

Antiviral assays

Activity of compounds against Human Immunodeficiency Virus type-1 (HIV-1) was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 µL of RPMI containing $1x10^4$ MT-4 were added to each well of flat-bottom microtitre trays containing 50 µL of RPMI, without or with serial dilutions of test compounds. Then, 20 µL of an HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4-day incubation, the cell viability was determined by the MTT method.

Stability tests in Cell Culture Medium

100 µM solutions of **8a-e** or **9**, prepared in RPMI supplemented with 10% FCS, 100 units/mL penicillin G and 100 µg/mL streptomycin, were incubated at 37 °C with shaking. To follow the rate of hydrolysis into MC-1220, 20 µL samples of the various prodrugs, collected at indicated times (Fig. 1), were injected on an SSODS2 reverse phase HPLC column, eluted with water/ethanol (40% water) at a flow rate of 0.6 mL/min and detected by UV at 260 nm.

Conclusions

The synthesis of the microbicide drug MC-1220 from easily available 4,6-dichloro-*N,N*,5-trimethylpyrimidin-2-amine was reduced to only three steps and was performed as a one-pot synthesis in high yield. Phosporamidate prodrugs of MC-1220 were synthesized and found to have almost the same potency of the parent drug against HIV-1. This was not the case for the glucoside prodrug, which failed to hydrolyze to MC-1220 in cell-free RPMI, even after 96 hours incubation. As far as we know, this is the first time that a phosphoramidate prodrug is made from a biologically active heterocyclic compound, taking advantage of its ability to form an aryloxy leaving group, which we believe is the driving force when the parent drug is released from the corresponding phosphoramidate.

Acknowledgements

This work received funding from the European Community's Sixth Framework Programme under contract number LSHP-CT-2004-503162 (Selection and development of microbicides for mucosal use to prevent sexual HIV transmission/acquisition).

Notes and references

1 K. T. Savjani, A. K. Gajjar and J. K. Savjani, *ISRN Pharmaceutics*, 2012, Article ID 195727
2 J. M. Férriz and J. Vinsová, *Curr. Pharm. Des.,* 2010, **16**, 2033–2052.
3 C. McGuigan, A. Salgado, C. Yarnold, T. Y. Harries, E. De Clercq and J. Balzarini, *Antiviral Chem. Chemother*., 1996, **7**, 184–188.
4 S. Aquaro, O. Wedgwood, C. Yarnold, D. Cahard, R. Pathinara, C. McGuigan, R. Calio, E. De Clercq, J. Balzarini and C. F. Perno*, Antimicrob. Agents Chemother*., 2000, **44**, 173–177.
5 M. Slusarczyk, M. H. Lopez, J. Balzarini, M. Mason, W. G. Jiang,S. Blagden, E. Thompson, E. Ghazaly and C. McGuigan, *J. Med. Chem.,* 2014, **57**, 1531−1542
6 M. Caron, G. Besson, S. L.-D. Etenna, A. Mintsa-Ndong, S. Mourtas, A. Radaelli, C. Morghen, R. Loddo, P. La Colla, S. G. Antimisiaris and M. Kazanji, *Virology,* 2010, **405**, 225–233.
7 N. Stolte-Leeb, R. Loddo, S. Antimisiaris, T. Schultheiss, U. Sauermann, M. Franz, S. Mourtas, C. Parsy, R. Storer, P. La Colla and C. Stahl-Hennig, *Aids Res. Hum. Retrovir*., 2011 **27**, 933–943.
8 S. M. Fetherston, L. Geer, R. S. Veazey, L. Goldman, D. J. Murphy, T. J. Ketas, P. J. Klasse, S. Blois, P. La Colla, J. P. Moore and R. K. Malcolm, *J. Antimicrob. Chemother.,* 2013, **68**, 394–403.
9 Y. M. Loksha, E. B. Pedersen, R. Loddo, G. Sanna, G. Collu, G. Giliberti and P. La Colla, *J. Med. Chem*., 2014, **57**, 5169−5178.
10 D. Jochman, *Virus Res*., 2008, **134**, 171–185.
11 J. Ren and D. K. Stammers, *Virus Res*., 2008, **134**, 157–170.
12 C. Sahlberg and X.-X. Zhou, *Anti-Infect. Agents Med. Chem*., 2008, **7**, 101–117.
13 J. L. Medina-Franco, K. Martínez-Mayorga, C. Juárez-Gordiano and R. Castillo, Chem. Med. Chem., 2007, **2**, 1141–1147.
14 E. De Clercq, *Chem. Biodivers*., 2004, **1**, 44–64.
15 D. G. Prajapati, R. Ramajayam, M. R. Yadav, R. Giridhar, *Bioorg. Med. Chem.,* 2009, **17**, 5744–5762.
16 G. Sbardella, A. Mai, M. Artico, P. Chimenti, S. Massa, R. Loddo, M. E. Marongiu, P. La Colla and A. Pani, *Antivir. Chem. Chemother*., 2001, **12**, 37–50.
17 M. Artico, *Drugs Future*, 2002, **27**, 159–175.
18 O. J. D'Cruz and F. M. Uckun, *J. Antimicrob. Chemother*., 2006, **57**, 411−423.
19 S. M. Schader, M. Oliveira, R.-I. Ibanescu, D. Moisi, S. P. Colby-Germinario and M. A. Wainberg, *Antimicrob. Agents Chemother*., 2012, **56**, 751−756.
20 A. Mai, M. Artico, D. Rotili, D. Tarantino, I. Clotet-Codina, M. Armand-Ugon, R. Ragno, S. Simeoni, G. Sbardella, M. B. Nawrozkij, A. Samuele, G. Maga and J. A. Este, *J. Med. Chem*., 2007, **50**, 5412–5424.
21 S. Bartolini, A. Mai, M. Artico, N. Paesano, D. Rotili, C. Spadafora and G. Sbardella, *J. Med. Chem*., 2005, **48**, 6776–6778.
22 M. Radi, L. Contemori, D. Castagnolo, R. Spinosa, J. A. Este, S. Massa and M. Botta, *Org. Lett*., 2007, 9, 3157−3160.
23 W. Hofer, F. Maurer, H. J. Riebel, I. Hammann and W. Behrenz, *Ger. Offen*. 1977, DE 2620089.
24 Y. M. Loksha, D. Globisch, R. Loddo, G. Collu, P. La Colla and E. B. Pedersen, *Chem. Med. Chem.,* 2010, **5**, 1847–1849.
25 C. McGuigan and J. Balzarini, 2000, WO 2000018775.
26 M. Sokolov, V. I. Zakharov, E. P. Studentsov, *Russ. J. Gen. Chem.,* 2002, **72**, 806 –811.
27 Y. Tsuzuki, K. Tanaka, *Bull. Chem. Soc.* Japan, 1967, **40**, 1208–1211.
28 R. Pauwels, J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyster E. De Clercq, *J. Virol. Methods,* 1988, **20**, 309–321.