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Synthesis and Evaluation of Novel Amidate Prodrugs of PMEA and PMPA

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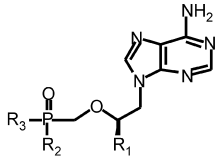
Abstract—Some novel amidate prodrugs of PMEA and PMPA have been synthesised and tested in vitro for their biological activity. Compound **5** in particular showed greatly enhanced antiviral potency compared with the parent nucleotide analogue. In vitro enzymatic studies and structure–activity relationships indicate that the degradation mechanism of such prodrugs may be the same as that described for the phosphoramidate triesters of nucleotide analogues. © 2001 Elsevier Science Ltd. All rights reserved.

Acyclic nucleoside phosphonates (ANPs) represent a class of nucleotide analogues in which a phosphonate group is linked to the alkyl side chain of various purines and pyrimidines. This class of nucleoside analogues possesses broad-spectrum antiviral activity, together with a high level of selectivity in vitro and in vivo.[1] The prototype compounds are: (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC, cidofovir), which is active against a wide variety of DNA viruses;[2] 9-(2-phosphonylmethoxyethyl)adenine (PMEA, Fig. 1, **2**, adefovir), which is active against retro-, herpes-[3] and hepadnaviruses,[4] and (*R*)-9-(2-phosphonylmethoxypropyl) adenine (PMPA, Fig. 1, **1**, tenofovir), which is active against retro- and hepadnaviruses.[5] Due to the structural nature of the P–C linkage, the ANPs are enzymatically stable and moreover they circumvent the first intracellular phosphorylation that is necessary for the activation of classical nucleoside analogues such as acyclovir. One of the disadvantages of the acyclic nucleoside phosphonates is the negative charge of the phosphonate moiety which significantly impairs their cellular uptake and causes low oral bioavailability. Their membrane transport is an active process that is considerably slower and less efficient than that of nucleoside analogues that cross the cell membrane by the nucleoside transport carrier system (e.g., for ddC) or by passive diffusion (e.g., for AZT). In order to improve the cellular uptake of the acyclic nucleoside phosphonates, ester derivatives have been synthesized that contain a lipophilic group attached to the phosphonate moiety. The bis(pivaloyloxy methyl)- [bis-(POM)] ester of PMEA (**3**) shows a >100-fold increase in cellular uptake, resulting in a markedly higher antiviral efficacy and cytotoxicity when compared to PMEA.[6,7]

However, the delivery of one molecule of the parent drug results in the liberation of two equivalents of potentially toxic formaldehyde and pivalinic acid.



Compds	R_1	R_2	R_3
1	CH_3	OH	OH
2	H	OH	OH
3	H	POM-O	POM-O
4	CH_3	POC-O	POC-O
5	CH_3	L-Ala-Me-ester	PhO
6	CH_3	Gly-Me-ester	PhO
7	CH_3	D-Ala-Me-ester	PhO
8	CH_3	L-Phe-Me-ester	pCl-PhO
9*	CH_3	L-Ala-Me-ester	pCl-PhO
10	CH_3	PhO	PhO
11	H	L-Ala-Me-ester	PhO
12	H	Gly-Me-ester	PhO
13	H	D-Ala-Me-ester	PhO

*±PMPA

Figure 1. Amidate prodrugs of PMEA/PMPA.

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Additionally, it has been shown that bis(POM) phosphotriesters were chemically unstable and highly susceptible to serum-mediated hydrolysis, factors which limit their potential utility for intracellular drug delivery. A modification of the bis(POM) approach is the bis(POC). This modification uses a carbonate diester within the masking group. The bis(POC) has been chosen as the carrier of choice for PMPA.[8] Similarly to the bis(POM), the bis(POC) was found to be highly unstable towards hepatic and blood esterases. In contrast to the bis(POM) approach, the bis(POC) avoids the generation of pivalinic acid. However, it still generates two equivalents of formaldehyde. As a result, given that bis(POM) and bis(POC) might not be the ideal prodrug solution to the ANPs, different prodrug strategies should be examined.

Here we report our work on the design, synthesis and biological evaluation of a new class of amidate prodrugs of PMPA and PMEA.[9]

The arylphosphoramidate triester approach has been successfully applied to a variety of nucleoside analogues such as d4T,[10] d4A[11] and ddA.[12] A typical example of a phosphoramidate prodrug is given by the d4T-5′-phenyl methoxyalaninyl phosphoramidate triester **15** (Fig. 2).

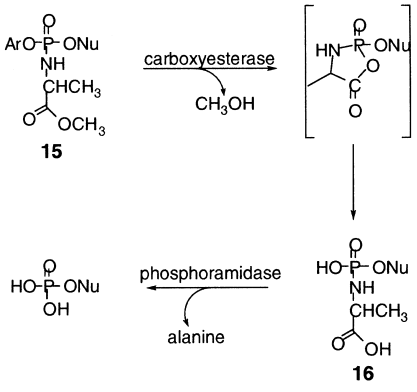

Figure 2. Metabolic activation of phosphoramidate prodrugs.

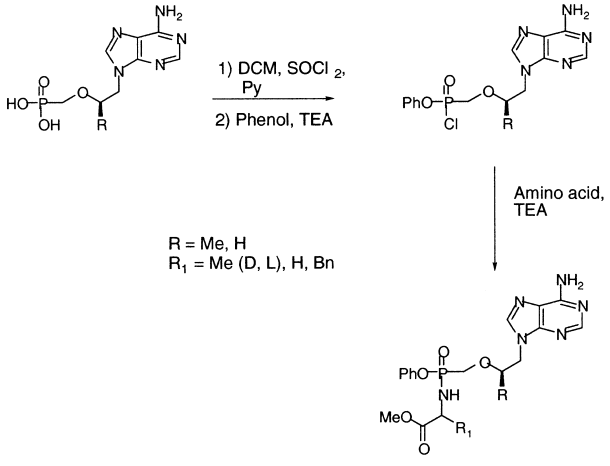

Figure 3. Synthetic route.

The degradation of the phosphoramidates is triggered by a carboxyl esterase-mediated cleavage of the methyl ester.[13] After subsequent cyclization and elimination of the phenol via a putative five-membered ring intermediate, a relatively stable aminoacyl phosphoramidate diester **16** is formed. A phosphoramidase is then believed to cleave the P–N linkage releasing the free nucleotide.[14] It has to be noted that the lability of P–N bonds in phosphonamidates compared to those in phosphoramidates is well documented.[15] The P–N bond in a phosphonamidate monoester, is labile and hydrolyzed faster than that in a phosphoramidate diester.[15] This suggests that if the amidate prodrugs of PMEA/PMPA were activated following a similar degradation process as described for the phosphoramidates, the P–N linkage of the aminoacyl phosphonamidate intermediate (i.e., **17**, **18**, Fig. 4) may undergo spontaneous hydrolysis in addition to an enzymatic mediated step. This may be a potential advantage of the phosphonamidates because the activation step would be triggered by a single esterase-mediated step and less dependent on the phosphoramidase activity. In addition, it has been shown that the stability of the phosphoramidate towards enzymatic degradation is affected by the nature of the amino acid used and by the nature of the carboxylic ester function at the amino acid moiety.[14] If a similar dependence is found for the amidate prodrugs of phosphonates PMEA and PMPA, this would be a potential advantage over the bis(POM)/bis(POC) prodrugs, because it may be possible to more readily modulate the release of parent drug. For these reasons amidate prodrugs of ANPs may represent an interesting alternative to the previously reported prodrug approaches. As a result, our first efforts were aimed to the synthesis of the phenyl methoxyalaninyl phosphoramidate diester of PMPA and PMEA and to the evaluation of their in vitro biological activity. The synthetic procedure proposed was based on the conversion of the parent nucleoside phosphonate analogues into their reactive phosphonodichloridates followed by a series of two couplings (Fig. 3). The chemistry of phosphonochloridates (their generation and coupling) has been described as idiosyncratic and the success in complex systems is possible only when mild conditions are employed.[16]

PMPA and PMEA were converted in mild conditions to their corresponding dichloridate using thionyl chloride as chlorinating agent and pyridine as base and catalyst. The reactions were checked for completion by ^{31}P NMR and the crude product was used for the next step. From

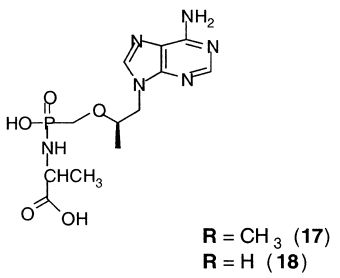

Figure 4.

the phosphonodichloridates a series of two subsequent couplings with the phenol and then the amino acid methyl ester in the presence of triethylamine yielded the desired target compounds. The order in which the couplings were made appeared to be essential. An additional problem was presented by the instability of the phenyl-phosphonochloridate during the reaction work-up.

As a result, a second chlorination reaction was often required. A final purification by column chromatography yielded the desired compounds with overall yield of 5% for **5** and **11**.[17] Compounds **5** and **11** were tested for their biological activity in vitro. Both amidate prodrugs displayed an enhanced antiviral activity when compared with the underivatized acyclic nucleoside phosphonate analogues (Table 1). The amidate **5** was found to be 50–100 times more potent than PMPA (**1**) against HIV-1 in MT4 cells whilst the corresponding amidate of PMEA (**11**) was 30–50-fold more potent than PMEA. Moreover, the selectivity index (SI) of **5** showed a significant enhancement (ca. 25 times) compared with **1** in MT4 cells. These results support the notion that phosphoramidate prodrugs of ANPs may be of medicinal and therapeutic interest. Following these results, we tried to establish whether the degradation mechanism of such amidate prodrugs was the same as their phosphoramidate counterparts. Compounds **5** and **11** underwent our ^{31}P NMR based carboxyl esterase assay[18] to see whether the starting phosphonamidate prodrugs were processed by the enzyme, releasing the corresponding aminoacyl phosphonamidate monoesters. Both substrates showed, at time 0, two ^{31}P NMR signals, one for each phosphate diastereoisomer. As the enzymatic reaction proceeded, these two signals of the starting material decreased with time with a calculated half-life of 100 and 37 h for compounds **5** and **11**, respectively.

Simultaneously another ^{31}P NMR signal appeared at 18.1 ppm in the case of **5**, and at 17.7 in the case of **11**. Likely, as happened with the phosphoramidates under the same conditions, these ^{31}P NMR signals correspond to the alaninyl phosphoramidate monoesters of R-PMPA (**17**, Fig. 4) and PMEA (**18**) suggesting that the first enzymatic step in the degradation of the amidate prodrugs may be the same as described for the nucleoside phosphoramidates. Interestingly, no degeneration to the free ANP (**1**, **2**) was observed by ^{31}P NMR after 48 h under the conditions used for the enzymatic assay. This indicates that compounds **17** and **18** are relatively stable. One of the clearest structure–activity relationships regarding the phosphoramidate prodrugs of d4T, was that the L-alanine was the preferred amino acid and that inversion of the chiral center at the amino acid to the D-series was detrimental for biological activity with a 30-fold reduction in antiviral potency.[19] The reason for such a strong specificity toward the L-configuration was due to the fact that in order to release the free nucleotide, the aminoacyl phosphoramidate diester of d4T had to undergo a phosphoramidase-mediated P–N cleavage.

In the attempt to see whether the phosphonamidates had similar SARs to the phosphoramidates, the glycine- (**6**, **12**), D-alanine- (**7**, **13**) and L-phenylalanine- (**8**) phosphonamidates of PMEA and PMPA were synthesized and tested for comparison. In addition, also the aryl substituted **9** of the racemic PMPA was prepared. Interestingly, from the data shown in Table 1, it appeares that also for the phosphonamidates, the L-alanine is the preferred amino acid. It is notable that the D-alaninyl phosphonamidates (**7**, **13**) are, on average, 5–30 times (for the PMPA derivative) and 30–60 times (for the PMEA derivative) less potent than the corresponding L-alaninyl phosphonamidates, as it was noted for the phosphoramidates of d4T. Similar results were obtained for the glycine derivatives **6** and **12**. This suggests that also in the case of the phosphonamidates, the P–N cleavage may be enzymatically driven rather than by a spontaneous chemical hydrolysis.

In conclusion, the data of biological activity and stability towards esterase are in general agreement and indicate that compounds **5** and **11** act as tripartate prodrugs that enhance the antiviral potency of PMPA and PMEA. The mechanism of degradation of the phosphonamidates may follow the same two enzymatic steps involved in the degradation of the phosphoramidates. Similar SARs were found for the phosphonamidates of PMEA and PMPA as earlier noted for nucleoside analogues such as d4T.

Table 1. Anti-HIV activity of test compounds

Compds	EC_{50} (μM)[a] HIV-1 MT4	EC_{50} (μM)[a] HIV-2 MT4	CC_{50} (μM)[b] MT4	EC_{50} (μM)[a] HIV-1 CEM	EC_{50} (μM)[a] HIV-2 CEM	CC_{50} (μM)[b] CEM
1	2.3	1.4	197	3.67	3.67	≥250
5	0.029	0.026	71.4	0.053	0.090	27
6	0.58	0.15	102	0.23	0.31	88.5
7	0.99	0.65	213	0.5	0.38	125
8	0.04	0.07	64	0.07	0.06	26
9	0.15	0.26	57	0.2	0.12	106
10	6.2	4.9	≥250	4.0	4.0	>250
2	7.0	7.5	144	7.0	10	69
11	0.23	0.15	5.1	0.12	0.20	3.7
12	3.6	4.8	120	5.6	4.5	80
13	15	7.2	120	5.0	6.0	67

[a]EC_{50}, 50% effective concentration.
[b]CC_{50}, 50% cytotoxic concentration.

Acknowledgements

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17. Synthetic and brief spectroscopic data on compound **5**: PMPA was suspended in anhydrous dichloromethane. After two consecutive additions of thionyl chloride (3.9 equiv) and of anhydrous pyridine (3.5 equiv), the mixture was left stirring under nitrogen for 30 min at room temperature. The solvent was removed under vacuo and the residue was co-evaporated with anhydrous dichloromethane. The residue was redissolved in anhydrous dichloromethane. To the stirring solution, a dropwise addition of a solution of phenol (0.9 equiv), freshly distilled triethylamine (1 equiv) and anhydrous dichloromethane was made at −78 °C. The reaction mixture was stirred for 16 h. The solvent was then removed under vacuo and redissolved in anhydrous dichloromethane. A second chlorination reaction was made as described above. Alanine methylester hydrochloride (1 equiv) and anhydrous dichloromethane were added to the residue. To the stirring solution, a dropwise addition of a solution of triethylamine (2 equiv) and anhydrous dichloromethane was made at −78 °C. The resulting mixture was stirred under nitrogen for 16 h upon warming to room temperature. The reaction was monitored by TLC (silica gel; eluent: 10% methanol in dichloromethane). The solvent was then removed under vacuo and the residue purified by column chromatography (silica gel; eluent: 5% methanol in dichloromethane) obtaining the desired product as colorless gums; yield 5%; δ_P ($CDCl_3$) 21.96, 23.60; δ_H 1.29 (6H, m, Ala-C$\underline{H}_3$, PMPA-C$\underline{H}_3$), 3.71 (4H, m, OC$\underline{H}_3$, Ala-C$\underline{H}$), 4.05 (5H, m, C$\underline{H}_2$-P, C$\underline{H}_2$-N, Ala-N$\underline{H}$), 4.41 (1H, m, PMPA-C$\underline{H}$), 5.87 (2H, bs, N$\underline{H}_2$), 7.04 (1H, m, Ph), 7.24 (4H, m, Ph), 8.03 (1H, bs, H-2), 8.39 (1H, m, H-8); δ_C 17.01, 17.08 (PMPA-$\underline{C}H_3$), 21.74 (Ala-$\underline{C}H_3$), 48.68, 48.84 ($\underline{C}H_2$-N), 49.82 (PMPA-$\underline{C}H$), 51.24 (Ala-$\underline{C}H$), 52.82 (O$\underline{C}H_3$), 63.75, 65.83 ($\underline{C}H_2$-P), 119.64 (C-5), 120.87, 120.93, 120.99 (Ph), 125.39 (Ph), 130.08 (Ph), 142.24 (C-8), 150.35, 150.59 (Ph), 153.26 (C-2), 155.71 (C-4), 160.74 (C-6), 174.47 (Ala-$\underline{C}O$); MS *m/e* FAB 471.1523 (MNa^+ $C_{19}H_{25}N_6O_5NaP$ requires 471.1522).
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