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Synthesis and stability studies of phosphonoformate–amino acid conjugates: a new class of slowly releasing foscarnet prodrugs

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Abstract—Prodrugs of phosphonoformic acid (PFA), an anti-viral agent used clinically as the trisodium salt (foscarnet), are of interest due to the low bioavailability of the parent drug, which severely limits its utility. Neutral PFA triesters are known to be susceptible to P–C bond cleavage under hydrolytic de-esterification conditions, and it was previously found that P,C-dimethyl PFA P–N conjugates with amino acid ethyl esters did not release PFA at pH 7, and could not be fully deprotected under either acid or basic conditions, which led, respectively, to premature cleavage of the P–N linkage (with incomplete deprotection of the PFA ester moiety), or to P–C cleavage. Here we report that novel, fully deprotected PFA-amino acid P–N conjugates **4** can be prepared via coupling of C-methyl PFA dianion **2** with C-ethyl-protected amino acids using aqueous EDC, which gives a stable monoanionic intermediate **3** that resists P–C cleavage during subsequent alkaline deprotection of the two carboxylate ester groups. At 37 °C, the resulting new PFA-amino acid (Val, Leu, Phe) conjugates (**4a**–**c**) undergo P–N cleavage near neutral pH, cleanly releasing PFA. A kinetic investigation of **4a** hydrolysis at pH values 6.7, 7.2, and 8.5 showed that PFA release was first-order in [**4a**] with respective $t_{1/2}$ values of 1.4, 3.8, and 10.6 h.

Foscarnet, the trisodium salt of phosphonoformic acid (PFA), has been used as an antiviral agent[1–4] to treat AIDS-related cytomegalovirus infection, and also exerts an inhibitory action against HIV.[5] The antiviral effect of PFA is attributed to inhibition of viral nucleic acid polymerases.[6,7] Because of its multiple negative charges at physiological pH, PFA has low oral bioavailability; this poor ability to penetrate cell membranes has long been a major impediment to its overall effectiveness as an anti-viral drug.[8,9] Past efforts to address this shortcoming by prodrug strategies have included partial esterification of PFA,[8–13] including its incorporation into cyclic esters,[11] and the replacement of an oxygen atom in PFA by a sulfur atom.[14–16] It has been shown that all three acidic groups of PFA must be available for maximal nucleic acid polymerase inhibition activity, and thus the ultimate antiviral activity of PFA prodrugs wherein one or more of these groups has been derivatized by esterification (or amidation) is likely to depend on a hydrolytic activation process in vivo to release the active drug.[8] Thus, PFA–amino acid conjugates might be useful as prodrugs of foscarnet, provided that such compounds are synthetically accessible and facile cleavage of the PFA–amino acid linkage occurs in vivo. It was recently reported that a fully alkyl-protected, neutral P–N linked PFA–amino acid phosphoramide produced by coupling (MeO)(Cl)P(O)CO_2Me with an L-amino acid Et ester, decomposed with P–C cleavage under basic deprotection conditions.[17–19] The same workers found that acidic conditions applied to the same protected conjugate hydrolyzed its P–N linkage instead of dealkylating the PFA diester.[17] This perplexing result is not unanticipated, given that the P–C bond in PFA esters is known to be highly activated due to juxtaposition of the carbonyl and phosphoryl groups, and is highly susceptible to cleavage under hydrolytic conditions to produce a phosphate or phosphite, depending on whether the water nucleophile attacks at carbon or phosphorus.[20–24] We reasoned that an intermediate product from condensation between C-methyl PFA and a C-alkyl-protected amino acid should be stabilized to unwanted P–C bond cleavage at high pH by virtue of its retention of one negative charge on the phosphonate group, thereby permitting alkaline deprotection of both carboxylate esters.

We report here successful application of this simple strategy to synthesis of the first examples of PFA–amino acid conjugates (**4**), and describe the surprisingly

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facile release of PFA from these isolable prodrugs at physiological pH and temperature.

To circumvent the decomposition observed previously in attempted hydrolytic deprotection of a neutral L-amino acid C-ester-PFA P-diester P–N conjugate,[17] we investigated coupling of a dianionic PFA C-monoester such as **2** with a C-blocked amino acid. The single negative charge remaining on the phosphoryl group in the product (**3**) was expected to impede P–C bond cleavage during subsequent alkaline hydrolysis of the protecting alkyl groups. To form the P-N linkage, we chose EDC-promoted coupling of **2** with C-ethyl protected L-amino acids (Val, Leu, or Phe) in aqueous solution under neutral to slightly alkaline conditions.[25] The intermediate **2** was obtained by quantitative and regioselective silyldealkylation of the *P,P*-dimethyl groups of trimethyl PFA with bromotrimethylsilane (BTMS).[26,27] The amidation reaction was followed by ^{31}P NMR, which revealed replacement over several hours of the singlet resonance of **2** by a doublet due to C**H**–N–**P** coupling. The condensation reaction went to completion without detectable formation of double condensation by-product, even with excess EDC and amino acid. The intermediate reaction mixture was processed to remove unreacted amino acid and NaCl, and the intermediate product (**3a**–**c**) was then hydrolyzed with NaOH to yield the target PFA conjugates (**4a**–**c**). Gratifyingly, **3** was deprotected under these conditions without any trace of P–C or P–N bond cleavage. Careful control of pH, and appropriate choice of washing/extraction solvents were important for success. Specifically, the coupling reaction was done within a pH range of 7.0–7.5; excess amino acid and NaCl were removed from the reaction mixture by extraction with EtOAc and acetone, successively, after adjustment of the pH to 9.5; dealkylation of **3a**–**c** was optimized at pH ~12.5; and purification of the final products **4a**–**c** was accomplished by removal of remaining EDC/by-products with acetone, followed by washing with MeOH or EtOH to remove excess NaOH. The structural integrity of the conjugate during work up and purification was monitored by the C**H**–N–**P** ^{31}P NMR doublet (δ_P 3–4, $^3J_{PH=}$ ~8.5 Hz) (Scheme 1).

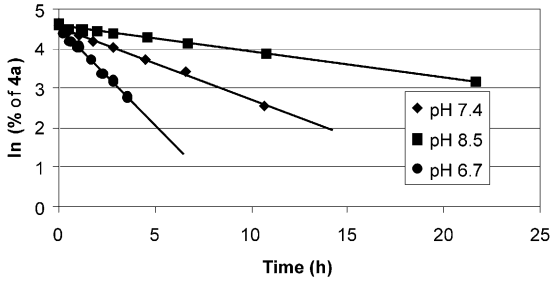
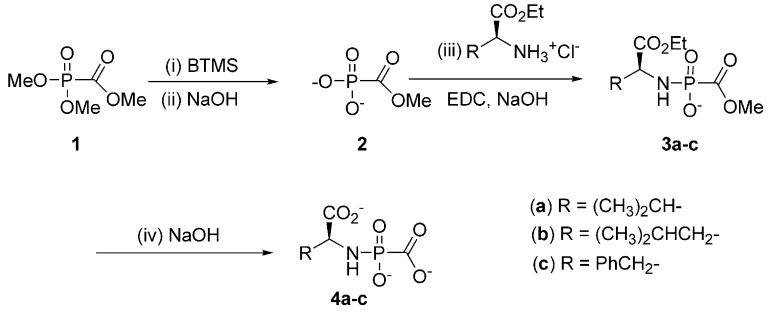


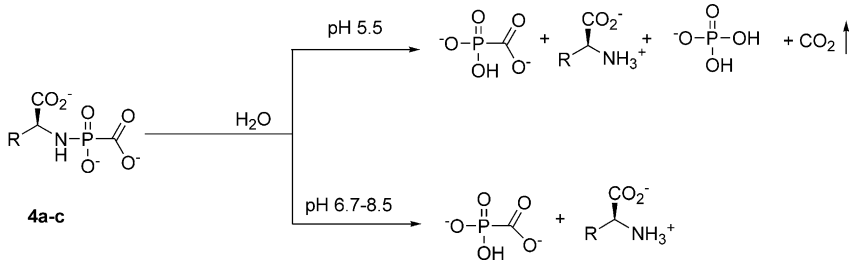
Figure 1.

The structure and purity of the products were confirmed by NMR (^{1}H, ^{13}C, and ^{31}P), combustion microanalysis and HRMS.[28] The compounds were stable at room temperature in the solid state.

The potential of the conjugates **4** as prodrugs depends on their ability to release the active drug PFA under mild conditions. Interestingly, they all cleanly released PFA and amino acid at physiological pH and temperature, *with no P–C bond cleavage observed*. The kinetics of pH-dependent release of PFA from **4a** was studied at several pH values near neutrality (6.7, 7.4, 8.5; all at 37 °C) by ^{31}P NMR. P–N bond cleavage liberating PFA was exclusively observed in a process characterized by pseudo-first order kinetics, with $t_{1/2}$ values of 1.4, 3.8 and 10.6 h, respectively (Fig. 1 and Scheme 2). The other two analogues (**4b**–**c**) also cleanly released the parent drug within this pH range (data not shown). All conjugates were found to be unstable at low pH; at pH



Scheme 1.



Scheme 2. Hydrolytic pathways of the conjugates at different pH values.

5.5 they underwent competitive P–C and P–N bond cleavage producing phosphate along with PFA.[29]

In conclusion, we have elaborated a practical method for synthesis and isolation of deprotected P–N linked PFA–amino acid conjugates. These phosphonopeptide analogues are good candidates for evaluation as prodrugs of PFA because they release the drug quantitatively under physiological conditions, even in the absence of amidases. These conjugates or related compounds might also be susceptible to active transport by membrane peptide transporters.[30–33]

Acknowledgements

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28. Synthesis of *N*-[(hydroxycarbonyl)hydroxyphosphinyl]-L-Valine, Trisodium Salt, **4a**: to 2.00 mL trimethyl phosphonoformate (14.87 mmol) stirred magnetically under nitrogen was added slowly 6.00 mL BTMS (45.46 mmol). The reaction mixture was stirred overnight at room temperature and excess BTMS was removed by rotary evaporation. The residue was immediately treated with 1 N NaOH to obtain a pH of 9.4. Volatiles were removed by rotary evaporation, and the residue was lyophilized to obtain a white powder, **2** (2.33 g), which was used in coupling reactions without further purification. NMR: δ_H (D_2O) 3.65 (s); δ_P (D_2O): −2.85 (s). In the coupling reaction, 200 mg of **2** (1.09 mmol) in 7 mL H_2O was treated with 590 mg L-valine ethyl ester hydrochloride (3.25 mmol) and 575 mg EDC (3.00 mmol). Then 1 N NaOH was added to adjust the pH to 7.5, and the mixture stirred overnight at room temperature; conversion to coupled product **3a** was quantitative by ^{31}P NMR. The reaction mixture pH was readjusted to 9.5 (1 N NaOH) then extracted with EtOAc (3×50 mL) to remove excess amino acid. The aqueous phase was then evaporated to dryness by rotary evaporation, and the conjugate separated from NaCl by extraction with acetone. The acetone phase was evaporated to dryness and the residue dissolved in 7 mL H_2O, then hydrolyzed by adding 5 N NaOH until the pH was 12.5. Reaction was complete at room temperature within 1/2 h. The reaction mixture was then evaporated to dryness and washed repeatedly with acetone and MeOH successively until a constant pH was attained (pH 9.5), then washed once again with acetone to leave **4a** as a white powder (139 mg, 38% yield from **1**). NMR: δ_H (D_2O): 3.17 (1H, dd, J = 9.3, 5.1 Hz), 1.63 (1H, m), 0.73 (3H, d, J = 7.3 Hz), 0.72 (3H, d, J = 6.9 Hz); δ_C (D_2O): 180.3 (d, $^3J_{CP}$ = 3.2 Hz), 179.4 (d, $^1J_{CP}$ = 215 Hz), 60.3 (d, $^2J_{CP}$ = 8.4 Hz), 30.3 (d, $^3J_{CP}$ = 3.9 Hz) 16.7, 15.3; δ_P (D_2O): 3.7 (d, $^3J_{PH}$ = 9.4 Hz). HRFAB-MS: *m*/*z* 267.9973 (M-Na)$^-$, calcd for $C_6H_9O_6NPNa_2$: 267.9963. Anal calcd for $C_6H_9NO_6PNa_3 \cdot H_2O$: C 23.32, H 3.59, N 4.53; found: C 23.29, H 3.36, N 4.37. Synthesis of *N*-[(hydroxycarbonyl)hydroxyphosphinyl]-L-Leucine, Trisodium Salt, **4b:** Using the procedure described for **4a**, 150 mg (0.82 mmol) of **2** produced 89 mg of **4b** (31% yield from **1**). NMR: δ_H (D_2O): 3.37 (1H, m), 1.39 (1H, m), 1.21 (2H, m), 0.63 (6H, m); δ_C (D_2O): 184.7 (d, $^3J_{CP}$ = 3.6 Hz), 182.2 (d, $^1J_{CP}$ = 220 Hz), 56.9 (d, $^2J_{CP}$ = 8.4 Hz), 46.2 (d, $^3J_{CP}$ = 4.0 Hz), 25.6, 23.5, 23.4; δ_P (D_2O): 3.4 (d, $^3J_{PH}$ = 9.1 Hz). HRFAB-MS: *m*/*z* 282.0129 (M-Na)$^-$, calcd for $C_7H_{11}NO_6PNa_2$ 282.0120. Anal calcd for $C_7H_{11}NO_6Na_3P.2H_2O$: C 24.65, H 4.43, N 4.11 found: C 24.70, H 4.11, N 4.04. Synthesis of *N*-[(hydroxycarbonyl)-hydroxyphosphinyl]-L-phenylalanine, Trisodium Salt, **4c**: Using the procedure described for **4a**, except coupling was done at pH 7.0, and purification steps were modified as specified below. Unlike **4a** and **4b**, this compound was partially soluble in MeOH, therefore washing with this solvent to remove excess base could not be applied. The product was instead washed repeatedly with acetone fol-

lowed by 1:1 EtOH/acetone until pH of the residue dissolved in H_2O was a constant (9.8); it was then washed with acetone, leaving **4a** as a white powder (281.0 mg, 43% yield from **1**). NMR: δ_H (D_2O): 7.10 (5H, m), 3.67 (1H, m), 2.83 (1H, m), 2.63 (1H, m); δ_C (D_2O): 182.5 (d, $^1J_{CP}=215.5$ Hz), 182.3 (d, $^3J_{CP}=3.8$ Hz), 139.3, 131.2, 129.8, 127.9, 59.3 (d, $^2J_{CP}=5.3$ Hz), 42.6; δ_P (D_2O): 3.2 (d, $^3J_{PH}=8.3$ Hz). HRFAB-MS: *m*/*z* 315.9963 $(M-Na)^-$, calcd for ($C_{10}H_9NO_6PNa_2$), 315.9963. Anal calcd for $C_{10}H_9NO_6PNa_3{\cdot}2H_2O$: C 32.02, H 3.49, N 3.73; found: C 32.44, H 3.16, N 3.75.

29. Hydrolysis kinetics of **4a**: Buffer solutions with pH 6.7, 7.4 or 8.5 (0.6 M borate–boric acid buffer) were prepared. **4a** (10 mg) in an NMR tube was dissolved in 0.5 mL buffer solution plus two drops of D_2O, and immediately placed in an thermostat at 37 °C. The progress of hydrolysis was monitored by ^{31}P NMR at suitable intervals. At the given pH values, PFA was the only phosphorus containing product. The product was determined by comparison (^{31}P NMR) with authentic PFA. The % of **4a** remaining was calculated from the relative height of the NMR signal versus PFA produced during hydrolysis. The half-lives were calculated from the slopes of linear plots of the logarithm of the remaining conjugate **4a** versus time. The stability of **4a** was also studied at pH 5.5 using acetate buffer (0.6 M). Within 20 min, the conjugate underwent complete decomposition to PFA and phosphate with the evolution of CO_2. PFA and phosphate were identified by comparing ^{31}P NMR after spiking with the authentic compounds. The other conjugates **4b–c** also released the parent drug PFA in the neutral pH range.
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