Bioorganic & Medicinal Chemistry Letters, Vol.1, No.11, pp. 653-658, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

PHOSPHONAMIDATES AND PHOSPHONAMIDATE ESTERS AS HIV-1 PROTEASE INHIBITORS

Donald A. McLeod[†], Ross I. Brinkworth[‡]
Jon A. Ashley[†], Kim D. Janda[†*], and Peter Wirsching[†]

[†]The Scripps Research Institute, Department of Molecular Biology and Chemistry,
10666 North Torrey Pines Road, La Jolla, California 92033, and
[‡]The Centre for Drug Design and Development,
The University of Queensland, Qld 4072, Australia

(*Received* 23 *September* 1991)

Abstract - Simple dipeptides incorporating phosphonamidate and phosphonamidate ester isosteres for the scissile peptide bond are modest inhibitors of the HIV-1 protease. Their synthesis and inhibition studies are described.

The human immunodeficiency virus type-1 protease (HIV-1 PR) is a member of the family of aspartyl retroviral proteases and plays an essential role in viral replication.[1] It is widely recognized as a potential target for chemotherapy of the acquired immunodeficiency syndrome (AIDS).[2] One strategy to inhibit the mature HIV protease, and aspartyl proteases in general, has been based on the replacement of the scissile peptide bond at the substrates cleavage site with nonhydrolyzable transition-state analogue moieties.[3]

We recently began investigating phosphonamidates as a new class of inhibitors of aspartyl proteases, in particular the HIV protease. Phosphonamidates have been very effective inhibitors of metalloproteases (carboxypeptidase A, thermolysin);[4] however, because of their instability under acidic conditions, they have not been regarded as possible aspartyl protease inhibitors. Hence, the more stable tetrahedral phosphinates/phosphonates have been utilized.[5] Because of the importance of hydrogen bonding in enzyme-inhibitor interactions[6], it might be advantageous to substitute a stable form of the phosphonamidate for the scissile amide moiety to be cleaved. Consequently, we have begun to evaluate a series of phosphonamidates for their ability to inhibit HIV-1 PR.

Our strategy was to utilize what we term a "capped" phosphonamidate (phosphonamidate ester) to bridge the gap between simple phosphinates and the more sophisticated, yet pH sensitive, phosphonamidates. Figure 1 depicts the structure of this compound. The desirable features of this scissile bond replacement are that (1) it retains the nitrogen atom as an important recognition element of the scissile amide bond and depending on the R′ amino acid, an extra hydrogen bond, (2) the methylated phosphonamidate is <u>not</u> susceptible to acid hydrolysis, and (3) there is <u>no</u> overall charge

carried within this isosteric replacement, which may facilitate entry into the cell. Below, we elaborate upon these features and describe our preliminary results.

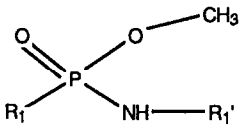
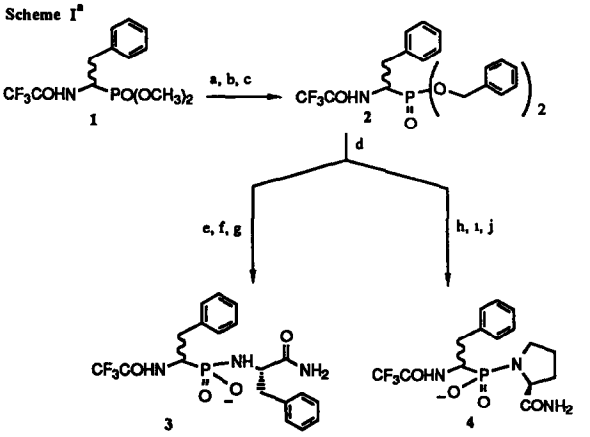


Figure 1. General structure of phosphonamidate esters, R_1 and R_1' represent amino acids.

Various investigators have sought to identify the target sites for the retroviral proteases[7]. For the HIV-1 PR, what emerged was a general preference for the P_1-P_1' positions to be hydrophobic residues. This provided a platform from which small synthetic oligopeptides could be constructed to form the basis of several *in vitro* assays for HIV-1 PR activity.[8] In addition, these peptides can serve as models for the design of potential inhibitors. Therefore, our plan was to test simple hydrophobic side chains at the P_1 and P_1' positions with the phosphonamidate moiety substituting for the scissile peptide bond. This appears somewhat naive because phosphonamidates are extremely unstable within an acidic environment where aspartyl proteases would exhibit optimum activity. However, because phosphonamidates have never been reported as aspartyl protease inhibitors, it seemed reasonable to examine this well known isosteric replacement, as a first approximation, provided a suitable enzymatic assay was available.

Expedient synthesis of the phosphonamidates **3** and **4** is shown in Scheme I[9]. The critical intermediate needed in obtaining either compound is phosphonate **1**[10]. The amino terminus in **1** is protected with a trifluoroacetyl moiety for synthetic convenience. Dimethoxyphosphonate **1** is converted to dibenzylphosphonate **2**. This step, while formally extending the synthesis, provides a functional group which can be removed efficiently under mild conditions without enlisting a final chromatographic purification step.

Scheme I[a]

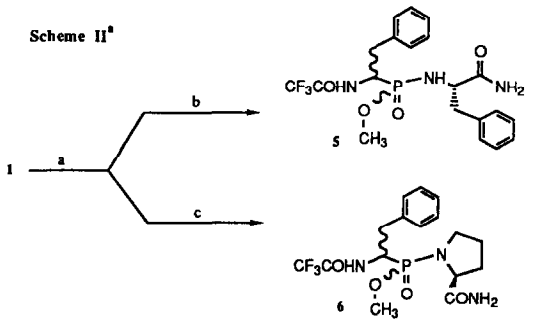


[a](a) TMSBr/CH_2Cl_2/40°C, 3h., 94%; (b) oxalyl chloride, neat, 45°C, 18h.; (c) benzyl alcohol (2 eq.), Et_3N (3 eq.), CH_2Cl_2, 2h., 60%; (d) PCl_5 (1.5 eq.) $CHCl_3$, 44°C, 18h.; (e) Phe-OCH_3 HCl (2 eq.), Et_3N (3 eq.), 18h R.T., 60% (f) H_2/Pd-C, 1.25 eq. $NaHCO_3$/MeOH, 1h., 95%; (g) NH_3/MeOH 0°C→ R.T. sealed vessel, 48h. 92%; (h) Pro-OCH_3 (3 eq), Et_3N (4 eq.), 18h., R.T., 55%; (i) NH_3/MeOH, 0°C→ R.T., 48H. sealed vessel, 80%; (j) H_2/Pd-C, 1.25 eq $NaHCO_3$, 1h, 98%.

The enzymatic assay chosen to investigate the inhibitory activity of phosphonamidates **3** and **4** was based on the continuous fluorometric assay originally developed by Toth and Marshall[11] which utilizes chemically synthetic HIV protease[19]. Under the assay conditions used[12], the acid labile phosphonates **3** and **4** were reasonably stable ($t_{1/2}$ of **3** and **4**, 30 hrs and 5 hrs respectively), thus allowing accurate determination of their IC_{50} and K_i values. The results (Table 1) indicated that the simple dipeptide phosphonamidate isosteres **3** and **4** can be effective inhibitors of the HIV-1 PR. We next turned our attention to stabilizing this moiety, if such compounds were to be effective for long periods of time under acidic conditions.

Simple monomethyl alkylation of the phosphonamidate moiety provided the necessary stabilizing element. We synthesized **5** and **6**, the "capped" versions of compounds **3** and **4**, respectively (Scheme II). Both **5** and **6** were prepared similarly to **3** and **4** using **1** as a common intermediate; however, their synthesis is greatly simplified by the fact that phosphonamidate ester deprotection is not required. These phosphonamidates were stable (less than 2% decomposition) over a period of one week at pH 3.5 (50 mM NaOAc, 23°C).

Scheme II[a]



[a](a) PCl_5 (1 5 eq.), 45^{0}C, 2h ; (b) Phe-NH_2, Et_3N (2 eq), $CHCl_3$, 16h., R T , 72%, (c) Pro-NH_2, (1 eq), Et_3N (4 eq), 18h. R T , 66%

Our findings (Table 1) demonstrated that the phosphonamidate methyl esters **5** and **6** retained comparable, albeit modest, potency to their "uncapped" counterparts **3** and **4**. It is interesting to compare this to inhibition of the metalloprotease ACE, where metal coordination is implicated, by unalkylated and alkylated (i.e.-OET) phosphonamidates. Here, the latter show a 700 to 4000-fold increase in K_i values. We also found that there was very little difference in potency between the inhibitors studied (i.e., Phe-Phe vs. Phe-Pro)(Table 1). This in contrast to findings by Grobelny and Galardy in which they utilized phosphinic acid isosteres to inhibit HIV-1 PR.[5a] They presented data which suggested the P_1-P_1' position was very sensitive to change. A replacement of BZ-Phe-$\psi[PO_2^-CH_2]$Phe-Val-Val-NH_2 with BZ-Phe-$\psi[PO_2^-CH_2]$ Pro-Val-Val-NH_2 increased K_i by a factor of 6500-fold. Variance amongst these findings and ours may be the result of a different mode of binding of our inhibitors, future studies should shed light on these differences.

Table 1. Inhibition Constants for HIV Protease Inhibitors[20]

No.	IC_{50} (μM)	K_i (μM)[20a]
3	105.0 +/-28.8	45.0
4	121.3 +/-10.8	52.0
5	184.0 +/-11.0	80.0
6	102.7 +/- 6.7	44.0

Potent inhibitors of aspartic proteinases[3a,b] have been designed by application of the transition-state analogue hypothesis[14] or through modification of naturally occurring aspartic proteinase inhibitors[15]. In all cases, the most potent aspartic proteinase inhibitors contain a hydroxyl group or phosphinate moiety that binds to the catalytic aspartic acid carboxyl groups in the enzyme inhibitor complex. These inhibitors are thought to mimic a reaction pathway intermediate for enzyme-catalyzed hydrolysis of amide bonds and analogues that lack either the critical hydroxyl or phosphinate group are weaker inhibitors by several orders of magnitude[16,5a,17]. We have shown that a tetrahedral phosphonamidate moiety can inhibit the HIV-1 PR. Yet, unexpected in light of traditional transition-state structure dogma, we have found that the phosphonamidate need not be in the form $\psi[PO_2^-NH]$ to be a protease inhibitor. Hence, while the tetrahedral geometry at the phosphorus atom is likely still of significance, its anionic charge may be of lesser importance[18].

Our future endeavors will be directed towards obtaining a therapeutically useful HIV-1 PR inhibitor. One strategy would be to extend either/both the C or N-terminus with amino acid residues or amino acid substitutes.[3a,b] Another approach to increase potency would be to obtain **5** and **6** in stereoisomerically pure form. Such studies should be helpful in designing phosphonamidate esters for the inhibition of other aspartyl proteases and might elucidate principles essential to the mechanism of action of these enzymes.

Acknowledgment: This work was financially supported by The Scripps Research Institute. We wish to thank Dianne Alewood and Bronwyn Garnham at the Centre for Drug Design and Development for the preparation of the HIV-protease.

Bibliography

1. Skalka, A.M., *Cell.* **1989**, 56, 911.
2. Johnston, M.I.; Allaudeen, H.S.; Sarver, N., *Tips.* **1989**, 10, 305.
3. (a) Rich, D.H.; Peptidase Inhibitors, *Comprehensive Medicinal Chemistry*; Sammes, P.G., Ed.; Pergamon Press: Oxford, 1990; Vol. 2, pp 391-441. (b) Rich D.H. *Proteinase Inhibitors*; Barret, A.J., Salvesen, G. Eds.; Research Monographs in Cell and Tissue Physiology; Elsevier Science Publ.; Amsterdam, 1986; pp 179-217 (c) Blundell, T.L.; Lapatto, R.; Wilderspin, A.F.; Hemmings, A.M.; Hobart, P.M.; Janley, D.E.; Whittle, P.J., *TIBS*, **1990**; pp 15, 425.
4. (a) Jacobsen, N.E.; Bartlett, P.A., *J. Am. Chem. Soc.* **1981**, 103, 654. (b) Bartlett, P.A.; Marlowe, C.K., *Biochem.* **1983**, 22, 4618. (c) Christianson, D.W.; Lipscomb, W.N., *J. Am. Chem. Soc.* **1988**, 110, 5560.
5. (a) Grobelny, D.; Wondrak, E.M.; Galardy, R.E.; Oroszlan, S., *Biochem. Biophys. Res. Comm.* **1990**, 169, 1111. (b) Bartlett, P.A.; Hanson, J.E.; Giannousis, P.P., *J. Org. Chem.* **1990**, 55, 6268. (c) Dreyer, G.B.; Metcalf, B.W.; Tomaszek, T.A.; Carr, T.J.; Chandler, A.C.; Hyland, L.; Fakhoury, S.A.; Magaard, V.W.; Moor, M.L.; Strickler, J.E.; Debouck, C.; Meek, T.D., *Proc. Natl. Acad. Sci. USA.* **1989**, 86, 9752. (d) Bartlett, P.A.; Kezer, W.B., *J. Am. Chem. Soc.* **1984**, 106, 4282.
6. (a) Bartlett, P.A.; Marlowe, C.K., *Science.* **1987**, 235, 569. (b) Grobelny, D.; Goli, U.B.; Galardy, R.E., *Biochem.* **1989**, 28, 4948. (c) Morgan, B.P.; Scholtz, J.M.; Ballinger, M.D.; Zipkin, I.D.; Bartlett, P.A., *J. Am. Chem. Soc.* **1991**, 113, 297.

7 (a) Pearl, L.H.; Taylor, W.R., *Nature* **1987**, 328 482. (b) Kotler, M.; Katz, R.A.; Danho, W.; Leis, J.; Skalka, A.M., *Proc. Natl. Acad. Sci. USA.* **1988**, 85, 4185.
8. (a) Billich, S.; Knoop, M.-T.; Hasen, J.; Strop, P.; Sedlacek, J.; Mertz, R.; Moelling, K., *J. Bio. Chem.* **1988**, 263, 17905. (b) Moore, M.L.; Bryan, W.M.; Fakhourg, S.A.; Magaard, V.W.; Huffman, W.F.; Dayton, B.D.; Meek, T.D.; Hyland, L.; Dreyer, G.B.; Metcalf, B.W.; Strickler, J.E.; Gorniak, J.G.; Debouck, C., *Biochem. Biophys. Res. Comm.* **1989**, 159, 420.
9. All new compounds were homogeneous by TLC or HPLC and were characterized by their satisfactory IR, NMR, and high-resolution FAB mass spectral data.
10. Fields, E.K., *J. Am. Chem. Soc.* **1952**, 74, 1528.
11. Toth, M.V.; Marshall, G.R., *Int. J. Peptide Protein Res.* **1990**, 36, 544.
12. Brinkworth, R.I.; Woon, T.C.; Fairlie, D.P., *Biochem. Biophys. Res. Comm.* **1991**, 176, 241.
13. (a) Galardy, R.E.; Kontoyianidou-Ostrem, V.; Kortylewicz, A.P., *Biochem.* **1983**, 22, 1990. (b) Elliot, R.L.; Marks, N.; Berg, M.J.; Portoghese, P.S., *J. Med. Chem.* **1985**, 28, 1208.
14. (a) Wolfenden, R., *Acc. Chem. Res.* **1972**, 5, 10. (b) Ibid, *Ann. Rev. Biophys. BioEng.* **1976**, 5, 271.
15. Boger, J.; Lohr, N.S.; Ulm, E.H.; Poe, M.; Blaine, E.H.; Fanelli, G.M.; Lin, T.-Y.; Dayne, L.S.; Schorn, T.W.; Lamonte, B.I.; Vassil, T.C.; Stabilito, I.I.; Veber, D.F.; Rich, D.H.; Boparai, A.S. *Nature* (London) **1983**, 303, 81.
16. Rich, D.H.; Northrop, D.B. Enzyme Kinetics in Drug Design: Implications of Multiple Forms of Enzyme on Substrate and Inhibitor Structure-Activity Correlations; Computer-Aided Drug Design; Perun, T.J.; Probst, C.L. Eds.; Marcel Dekker Inc.: New York, 1989; pp 185-250.
17. Rich, D.H., *J. Med. Chem.* **1985**, 28, 263.
18. The binding of the neutral form of phosphinate inhibitors has been proposed as a requirement for maximum inhibitor potency with aspartyl proteases. See references (6a), (6c) and Bartlett, P.A.; Marlowe, C.K.; Giannousis, P.P.; Hansen, J.E., *Cold Springs Harbor Symp. Quant. Biol.* **1987**, 52, 83-90.
19. Schneider, J.; Kent, B.H.S., *Cell* **1988**, 54, 363.
20. The enzyme assay has been described (reference 12) and is based on the spectrofluorometric assay of Toth and Marshall[11] using synthetic HIV protease[19] with the chief modification being that the assay buffer used was 100mM MES, pH 6.5. 10mM stock solutions of the inhibitors and substrate, 2-aminobenzoyl-Thr-Ile-Nle-(p-nitroPhe)-Gln-Arg-amide) were prepared in DMSO, with subsequent dilutions in buffer. Assays were performed in a 8X12 black microtitre plate (ICN-Flow Laboratories) with five concentrations of inhibitor (50μM-250μM) plus controls being tested in quadruplicate. Preincubation of enzyme plus inhibitor was performed at 37°C for 15 min. in a total volume per well of 180μl. The enzyme-catalyzed reaction was initiated by the addition of 20μl of 500μM substrate to give an assay concentration of 50μM. The K_m for the substrate under these assay conditions was 37.5 +/-5.0 μM. The reaction was left to proceed at 37°C for 5 min. then stopped by the addition of 100μl of 6.0 M guanidine HCl in buffer. The amount of protease in the assay was adjusted to produce < 10% cleavage of the substrate in 5 min. The plates were read in a Fluoroskan II fluorescent microtitre plate reader (ICN-Flow Laboratories). The fluorescence reading of the blanks (no cleavage) was typically 22.0 +/-2.0 at pH 6.5, with the control readings generally in the range 29.0-33.0. IC_{50} values for the inhibitors were calculated using a semi-automated Dixon plot calculation using the Microsoft Works spreadsheet.
[a]Estimated by Dixon analysis only, K_i = apparent inhibition constant.