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THE ROLE OF 4-PHOSPHONODIFLUOROMETHYL- AND 4-PHOSPHONO-PHENYLALANINE IN THE SELECTIVITY AND CELLULAR UPTAKE OF SH2 DOMAIN LIGANDS

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Abstract: Incorporation of 4-phosphonodifluoromethyl-phenylalanine (F_2Pmp) and 4-phosphono-phenylalanine into SH2 targeted peptides and peptidomimetic ligands was found to effect binding affinity and selectivity of these ligands toward the Src and Abl SH2 domains. Furthermore, dipeptide analogs containing these phosphonate amino acids were used to produce prodrugs with excellent cellular delivery and reconversion rates.

Understanding the details of how extracellular signals trigger intracellular events, especially nuclear processes such as protein synthesis, cell growth, or cell death, is a current area of intense research. The ability to modulate or interrupt specific signal transduction pathways potentially offers novel targets for therapeutic intervention.[1–3]

Many of the key signaling pathways inside cells are mediated by the phosphorylation (via kinases)[4,5] and/or dephosphorylation (via phosphatases)[6–8] of tyrosine residues found in various intracellular proteins. These changes in phosphorylation state are then responsible for the activation, inactivation or localization of these proteins, thus causing a signal to be propagated, amplified, interrupted, or localized to a specific region of the cell. A critical step in these pathways involves the recognition of these phosphorylated tyrosines in a sequence-specific manner by protein substructures or domains called Src Homology 2 (SH2) domains.[9–11] These domains are found in numerous intracellular proteins associated with cell signaling. Often these SH2 domain-containing proteins also possess a kinase or phosphatase domain. Thus, when associated with a phosphorylated tyrosine, they may then phosphorylate or dephosphorylate another tyrosine on an associated protein or even elsewhere in their own structure. In addition, several protein families are comprised of only SH2 and SH3 domains (SH3 domains bind poly-proline sequences) and serve as adapter proteins.[12] Thus, SH2 domains play distinct and pivotal roles in the assemblage of numerous multi-protein complexes and ultimately in the propagation of intracellular signals.

Our efforts have focused on the design of peptidomimetic and nonpeptide antagonists of phosphoprotein-SH2 binding. We targeted the Src SH2 domain based on the availability of several X-ray and NMR structures for this protein and its complexes with small phosphopeptides,[13–16] and because of its putative role in the development of cancer[17] and osteoporosis.[18] Using a structure-based drug design approach, we[19,20] and others [21,22] have previously reported on the conversion of an 11 amino acid peptide lead into simple tripeptides and more recently dipeptides,[23] which are nearly equipotent to the original 11-mer. However, despite excellent in vitro activity for several series of small peptides, these compounds can be expected to have poor cellular activity for two reasons. First, the phosphotyrosine (pTyr) moiety, which is expected to carry a -2 charge at physiological pH, is required to maintain high binding affinity. Since our targets are intracellular, these

compounds must cross the cell membrane, and in general, ionized compounds do not readily cross cell membranes via simple diffusion. The second limitation with phosphopeptides is the expected instability of the pTyr moiety. It is anticipated that specific and nonspecific phosphatases would rapidly dephosphorylate simple phosphopeptides, rendering them completely inactive.

An excellent solution to general lability of phosphotyrosines to phosphatases was provided by Burke and coworkers. These researchers adapted the well-known ability of $CH_2PO_3^{-2}$, and especially $CF_2PO_3^{-2}$, to mimic OPO_3^{-2} in many other biological systems,[24–26] to the area of signal transduction through the first synthesis and use of phosphonodifluoromethyl phenylalanine (F_2Pmp) as a mimetic for pTyr.[27] Burke et al. have further shown that peptides containing F_2Pmp in place of pTyr retain ability to bind to various SH2 domains and are, as expected, resistant to phosphatases.[28] In a subsequent study, they have further demonstrated that other F_2Pmp containing peptides can act as phosphatase inhibitors.[29]

The effectiveness of F_2Pmp as a replacement for pTyr depends on the particular SH2 domain to which the ligand is targeted. Some SH2 domains show decreased binding affinity to F_2Pmp-containing peptides, and others show equal or preferred binding for F_2Pmp over pTyr.[28] To evaluate the role of F_2Pmp in the binding of our compounds to Src and other SH2 domains, we synthesized a series of pTyr- and F_2Pmp-containing peptides. Two binding assays were used in this evaluation. The first is a radioligand displacement assay for the binding of ^{125}I-labeled **1** or **2** to Src and Abl, represented in Table 1 as **SSRC** and **SABL**, respectively. The second assay is a protein association assay which measures the disruption of the binding of ^{35}S-labeled SH2-GST fusion proteins of Src and Abl to the phosphorylated intracellular domain of the PDGF receptor by our ligands (see **Src** and **Abl** in Table 1).

Table 1. Binding and Specificity Data for pTyr, F_2Pmp, and Phe(PO_3H_2) Containing Peptides

		^{125}I Peptide		^{35}S Protein	
		SSRC	**SABL**	**Src**	**Abl**
		IC_{50} (μM)			
1	Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu	0.8	15	0.8	2.9
2	Glu-Pro-Gln-F_2Pmp-Glu-Glu-Ile-Pro-Ile-Tyr-Leu	5.5	1.8	11.7	13.5
3	Ac-pTyr-Glu-Glu-Ile-Glu	0.5	7.9	1.4	15
4	Ac-F_2Pmp-Glu-Glu-Ile-Glu	1.1	1.2	6.0	5.2
5	Ac- Phe(PO_3H_2)-Glu-Glu-Ile-Glu	6.7	22	ND	ND
6	Ac-pTyr-Glu-D-Hcy-NH_2	1.8	4.9	7.7	7.0
7	Ac-F_2Pmp-Glu-D-Hcy-NH_2	4.9	1.2	30	1.4
8	Ac-pTyr-Glu-NMe-(Pr-Chx)	0.8	4.0	9.0	16
9	Ac-F_2Pmp-Glu-NMe-(Pr-Chx)	3.4	1.7	30	2.1
10	Ac-Phe(PO_3H_2)-Glu-NMe-(Pr-Chx)	9.3	14	ND	ND
11	Ac-pTyr-Abu-NMe-(Pr-Chx)	5.5	15	26	100
12	Ac-F_2Pmp-Abu-NMe-(Pr-Chx)	7.3	1.6	~100	4.0
13	Ac-Phe(PO_3H_2)-Abu-NMe-(Pr-Chx)	6.1	22	>100	17

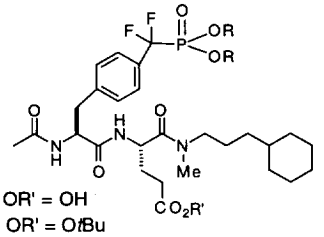
Hcy = homocyclohexylalanine, Pr-Chx = cylcohexylpropyl, Abu = *alpha*-aminobutyric acid, Phe(PO_3H_2) 4-phosphonophenylalanine, ND = Not Determined. The IC_{50}'s are the average of at least two duplicate determinations and were determined as described in Shahripour et al., see ref. 20.

Review of this data revealed that for any analogous pair of peptides the substitution of F_2Pmp for pTyr caused a loss in Src SH2 binding affinity, as was expected from previous reports.[28] This was true in both assays but more pronounced in the protein association assay, where typically a fourfold to fivefold decrease in Src SH2 binding affinity was observed. Surprisingly, incorporation of the F_2Pmp generally favored binding to the Abl SH2 domain over the Src SH2 domain. Furthermore, this effect increased as the size of the peptide decreased. For the 11-mers, **1** and **2**, and the pentapeptides, **3** and **4**, incorporation of F_2Pmp causes only an erosion of the normal Src selectivity, but for the tripeptide **6** and **7**, and the dipeptides **8** and **9**, and **11** and **12**, incorporation of F_2Pmp provides a maximum of 15- to 25-fold selectivity for Abl over Src.

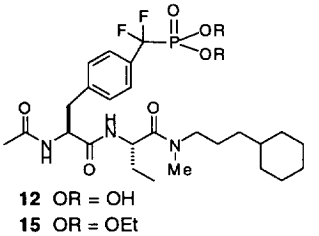
The apparent Abl selectivity of these compounds may be explained by unfavorable interactions of F_2Pmp with the Src SH2 domain. Specifically, the Src SH2 domain contains a cysteine in the phosphate binding pocket; in Abl the corresponding residue is a serine. It's possible that this cysteine thiol in Src forms poor steric and/or electrostatic interactions with the fluorine atoms of the F_2Pmp. Since Abl lacks this cysteine, such disfavored intermolecular interactions may not exist, but a more definitive answer must await future crystallographic analysis.

The utility of the F_2Pmp group to provide phosphatase resistant analogs of pTyr represents its prime advantage over pTyr. However, since F_2Pmp is still dianionic at physiological pH, it is expected that compounds containing F_2Pmp would show poor cellular activity due to poor cellular penetration. This expectation has led others to use invasive techniques such as microinjection[30] or cell permeabilization[31] to demonstrate the cellular activity of F_2Pmp-containing peptides. While these studies help establish the "proof-of-concept" that SH2 domains are possible therapeutic targets, they offer little encouragement for the ultimate development of a useful therapeutic agent. We reasoned, based on previous studies with nucleoside analogs,[32] that suitably protected esters of our F_2Pmp-containing ligands could provide a prodrug approach to cellular delivery.

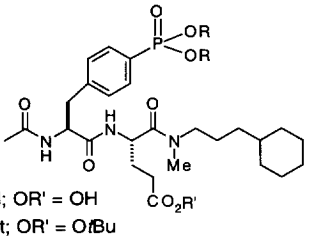
Figure 1. Structure of Dipeptides and Their Prodrug Analogs.



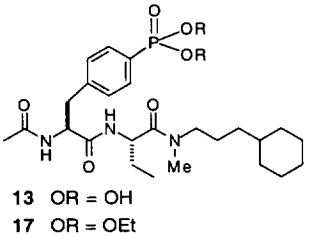
9 OR = OH; OR' = OH
14 OR = OEt; OR' = O*t*Bu
18 OR = OEt; OR' = OH
24 OR = OCH_2OPiv, OH; OR' = OCH_2OPiv



12 OR = OH
15 OR = OEt
19 OR = OEt, OH
25 OR = OCH_2OPiv, OH



10 OR = OH; OR' = OH
16 OR = OEt; OR' = O*t*Bu
20 OR = OCH_2OPiv, OH; OR' = OCH_2OPiv
22 OR = OCH_2OPiv; OR' = OCH_2OPiv

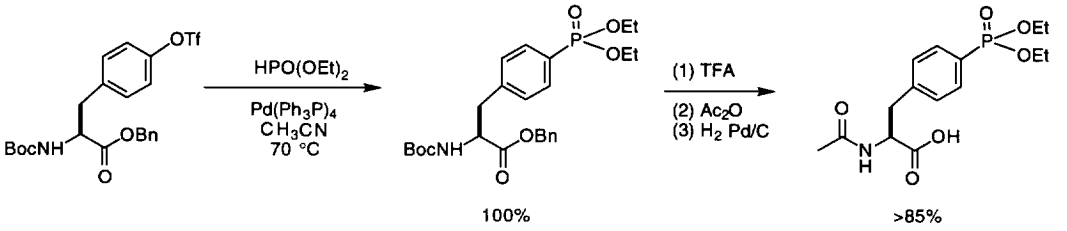


13 OR = OH
17 OR = OEt
21 OR = OCH_2OPiv, OH
23 OR = OCH_2OPiv

To address the problem of cellular delivery of our compounds, we chose the series of dipeptides (**9** and **12**, see Figure 1) with the hope that these compounds, with the phosphonate fully protected, would provide the best chance of attaining cellular uptake. Aside from the phosphonate group, these analogs were moderately to highly hydrophobic. The parent pTyr analogs in each of these series (**8** and **11**) showed moderate to good binding to the Src SH2 domain. Although replacement of the pTyr by F_2Pmp generally caused a decrease in affinity toward Src (see Table 1), this substitution was necessary to provide resistance to phosphatases.

Concurrent with our development of F_2Pmp containing peptides we also explored the use of 4-phosphono-phenylalanine containing peptides, as illustrated by **5**, **10**, and **13**. This series was chosen based on the expected ease of synthesis of various phosphonate prodrug esters from the readily available tyrosine-triflate and simple phosphite diesters via the reported palladium-catalyzed coupling reaction.[33] For simple dialkyl phosphite esters such as ethyl, this reaction proceeds in excellent yields to give the protected amino acid, which could be readily converted to the N-acetyl amino acid used in our studies, as indicated in Scheme 1. This protected arylphosphonate amino acid was then incorporated into two of our best series of dipeptide leads (see Figure 1). As can be seen in Table 1, dipeptides **10** and **13**, and pentapeptide **5** are generally less active than their pTyr or F_2Pmp analogs, but provide the potential for the synthesis of a variety of phosphonate diester prodrugs. However, attempts to extend this methodology to other phosphite esters with better prodrug potential[34] (e.g., diphenyl phosphite, or bis-trichoroethyl phosphite) provided little or no cross-coupled products. Thus, this coupling reaction appears to be particularly sensitive to electronic effects in the phosphite ester.

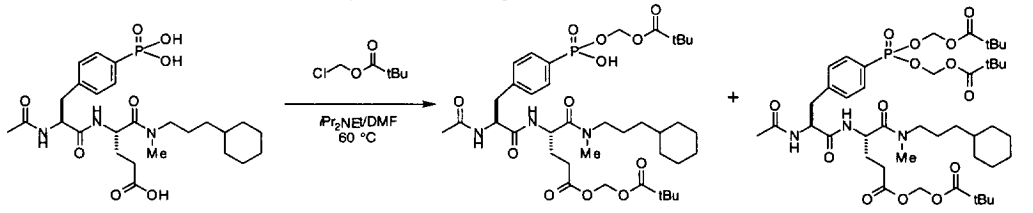
Scheme 1. Preparation of N-Acetyl-4-(diethyl phosphono)-phenylalanine



To test whether this simple masking strategy would work, we devised an ion-pair reversed-phase HPLC assay to monitor the uptake of these compounds directly into Balbc3T3 cells.[35,36] The results of this study (see Table 2) clearly show that the fully protected triester **14** and diester **17** readily get into cells to provide acceptable concentrations of the drug in these cells (as do **15** and **16**, data not shown). Interestingly, even the carboxylate mono-acid analog **18** achieved some cell uptake albeit at a much lower rate. However, no uptake could be observed for either the phosphonate mono- or diacids **9**, **10**, **12**, **13**, and **19** (data not shown).

The results of the uptake studies clearly indicated the potential utility of this prodrug approach to deliver phosphonate diesters into cells. It remained to identify diesters which would not only show good uptake but also reconvert to the active, parent diacids inside cells. As indicated above, the initial plan was to utilize the arylphosphonates to provide a wide range of phosphonate ester prodrugs, but synthetic difficulties in the key coupling step precluded the use of this approach. However, use of the acyloxymethyl esters championed by Farquhar et al. provided an excellent solution to our prodrug problem.[37] These prodrug derivatives proved to be surprisingly easy to generate from the di- or triacids and the commercially available chloromethyl pivaloate as exemplified in Scheme 2 for the arylphosphonate derivative **10**.

Scheme 2. Synthesis of Acyloxymethyl Ester Prodrugs



10 **20** **22**

However, when this methodology was applied to the F_2Pmp series, only the phosphonate mono-esters could be obtained (**24** and **25**), even under forcing conditions. Presumably the more acidic F_2Pmp, like pTyr, is difficult to alkylate under these conditions.[38] Fortunately, this difficulty would later provide us with an excellent solution to our drug delivery problem.

As expected, when these compounds were tested in our cellular uptake assay, the fully protected arylphosphonate di- and triesters (**22** and **23**) were again readily taken up into the Balbc3T3 cells, and moreover, were also readily converted via the short-lived, partially deprotected intermediates to the parent di- or triacids (see Table 2).[36] Surprisingly, in the F_2Pmp series the phosphonate mono-esters (**24** and **25**) were taken up into cells, albeit at a rate slower than the fully protected diesters (see Table 2). Even at this reduced rate **24** and **25** still accumulate to cellular concentrations equal to that seen for **14** and **23** within 30 minutes instead of 1-2 minutes.

Table 2. Uptake Rates and Reconversion for Selected Compounds in Balbc3T3 Cells.

Compound #	OR	OR′	Uptake Rate (nmol/min-sq cm) normalized to 100 μM	Reconversion
14	OEt	O*t*Bu	3.2	ND
17	OEt	NA	0.092 ± 0.008 (n = 3)	ND
18	OEt	OH	>0.002	ND
23	OCH_2OPiv	NA	6.6 ± 0.76 (n = 3)	Yes
25	OCH_2OPiv, OH	NA	0.01 ± 0.001 (n = 3)	Yes

The uptake rates were determined as reported in Surendran et al., See ref. 36, NA = Not Applicable, ND = Not Detected

However, in the arylphosphonate series, only the full protected di- and triesters (**22** and **23**) showed any significant uptake, and unlike the F_2Pmp series, the mono-phosphonate esters (**20** and **21**) showed no uptake (data not shown). The discovery that the mono-acyloxymethyl esters in the F_2Pmp series show good uptake provided us with a useful phosphonate prodrug. These analogs are highly water-soluble due to the free phosphonic acid, but appear to permeate cells and reconvert at a useful rate to provide adequate intracellular concentrations of the parent di- and triacids. Currently, we are exploring the further generality of these observations. One possible explanation for this difference in uptake behavior between the arylphosphonate and F_2Pmp series of mono-acyloxymethyl esters may be related to their differences in hydrophobicity, as their uptake seems to correlate with the log k'_{IAM} on an Immobilized Artificial Membrane-Phosphotidyl Choline (IAM.PC) HPLC column.[36,39]

With fully functional prodrugs in hand, we are currently focusing on the further testing of these compounds and others in a variety of signal transduction based cellular assays, in an attempt to demonstrate the effect of

blocking the binding of the Src SH2 domain to its natural phosphoprotein substrates. Results from these studies will be reported in elsewhere.

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References and Notes

1. Bridges, A. J. *Chemtracts: Org. Chem.* **1995**, *8,* 73.
2. Brugge, J. S. *Science* **1993**, *260,* 918.
3. Levitzki, A. *Curr. Opin. Cell Biol.* **1996**, *8,* 239.
4. Liu, D.; Wang, L. H. *J. Biomed. Sci.* **1994**, *1,* 65.
5. Levitzki, A.; Gazit, A. *Science* **1995**, *267,* 1782.
6. Kassim, S. K.; Wiener, J. R.; Berchuck, A.; Bast, R. C. *Cancer Mol. Biol* **1994**, *1,* 133.
7. Feng, G. S.; Pawson, T. *Trends in Genetics* **1994**, *10,* 54.
8. Tonks, N. K. *Adv. Pharmacol.* **1996**, *36,* .
9. Beattie, J. *Cell. Signalling* **1996**, *8,* 75.
10. Smithgall, T. E. *J. Pharmacol. Toxicol. Methods* **1995**, *34,* 125.
11. Botfield, M. C.; Green, J. *Annu. Rep. Med. Chem.* **1995**, *30,* 227.
12. Birge, R. B.; Knudsen, B. S.; Besser, D.; Hanafusa, H. *Genes Cells* **1996**, *1,* 595.
13. Waksman, G.; Kominos, D.; Robertson, S. C.; Pant, N.; Baltimore, D.; Birge, R. B.; Cowburn, D.; Hanafusa, H.; Mayer, B. J.; Overduin, M.; Resh, M. D.; Rios, C. B.; Silverman, L.; Kuriyan, J. *Nature(London)* **1992**, *358,* 646.
14. Waksman, G.; Shoelson, S. E.; Pant, N.; Cowburn, D.; Kuriyan, J. *Cell* **1993**, *72,* 779.
15. Waksman, G. *Cell. Mol. Biol.* **1994**, *40,* 611.
16. Xu, R. X.; Word, J. M.; Davis, D. G.; Rink, M. J.; Willard, D. H., Jr.; Gampe, R. T., Jr. *Biochemistry* **1995**, *34,* 2107.
17. Levitzki, A. *Anti-Cancer Drug Des.* **1996**, *11,* 175.
18. Hall, T. J.; Schaeublin, M.; Missbach, M. *Biochem. Biophys. Res. Comm.* **1994**, *199,* 1237.
19. Plummer, M. S.; Lunney, E. A.; Para, K. S.; Prasad, J. V. N. V.; Shahripour, A.; Singh, J.; Stankovic, C. J.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Sawyer, T. K. *Drug Design and Discovery* **1996**, *13,* 75.
20. Shahripour, A.; Plummer, M. S.; Lunney, E.; Para, K. S.; Stankovic, C. J.; Rubin, J. R.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Herrera, R.; Hubbell, S. E.; Saltiel, A. R.; Sawyer, T. K. *Bioorg. Med. Chem. Lett.* **1996**, *6,* 1209.
21. Rodriguez, M.; Crosby, R.; Alligood, K.; Gilmer, T.; Berman, J. *Lett. Pept. Sci.* **1995**, *2,* 1.
22. Gilmer, T.; Rodriguez, M.; Jordan, S.; Crosby, R.; Alligood, K.; Green, M.; Kimery, M.; Wagner, C.; Kinder, D.; Charifson, P.; Hassel, A. M.; Willard, D.; Luther, M.; Rusnak, D.; Sternbach, D. D.; Mehrotra, M.; Peel, M.; Shampine, L.; Davis, R.; Robins, J.; Patel, I. R.; Kassel, D.; Burkhart, W.; Moyer, M.; Bradshaw, T.; Berman, J. *J. Biol. Chem.* **1994**, *269,* 31711.
23. Plummer, M. S.; Lunney, E.; Para, K. S.; Shahripour, A.; Stankovic, C. J.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Herrera, R.; Hubbell, S.; Saltiel, A.; Sawyer, T. K. *Bioorg. Med. Chem.* **1997**, *5,* 41.
24. Blackburn, G. M. *Chem. Ind.* **1981**, *5,* 134.
25. Blackburn, G. M.; Perree, T. D.; Rashid, A.; Bisbal, C.; Lebleu, B. *Chem. Scr.* **1986**, *26,* 21.
26. Engel, R. *Chem. Rev.* **1977**, *77,* 349.
27. Burke, T. R., Jr.; Smyth, M. S.; Nomizu, M.; Otaka, A.; Roller, P. R. *J. Org. Chem.* **1993**, *58,* 1336.
28. Burke, T. R., Jr.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. *Biochemistry* **1994**, *33,* 6490.
29. Burke, T. R., Jr.; Kole, H. K.; Roller, P. P. *Biochem. Biophys. Res. Comm.* **1994**, *204,* 129.
30. Xiao, S.; Rose, D. W.; Sasaoka, T.; Maegawa, H.; Burke, T. R., Jr.; Roller, P. P.; Shoelson, S. E.; Olefsky, J. M. *J. Biol. Chem.* **1994**, *269,* 21244.
31. Wange, R. L.; Isakov, N.; Burke, T. R., Jr.; Otaka, A.; Roller, P. P.; Watts, J. D.; Aebersold, R.; Samelson, L. E. *J. Biol. Chem.* **1995**, *270,* 944.
32. Jones, R. J.; Bischofberger, N. *Antiviral Res.* **1995**, *27,* 1.
33. Thurieau, C.; Simonet, S.; Paladino, J.; Prost, J.-F.; Verbeuren, T.; Fauchere, J.-L. *J. Med. Chem.* **1994**, *37,* 625.
34. Starrett, J. E., Jr.; Tortolani, D. R.; Russell, J.; Hitchcock, M. J. M.; Whiterock, V.; Martin, J. C.; Mansuri, M. M. *J. Med. Chem.* **1994**, *37,* 1857.
35. Surendran, N.; Stankovic, C. J.; Stewart, B. H. *J. Chrom. B.* **1997**, *691,* 305.
36. Surendran, N.; *et al.* , *manuscript in preparation.*
37. Farquhar, D.; Khan, S.; Srivastva, D. N.; Saunders, P. P. *J. Med. Chem.* **1994**, *37,* 3902.
38. Srivastva, D. N.; Farquhar, D. *Bioorg. Chem.* **1984**, *12,* 118.
39. Pidgeon, C.; Ong, S.; Liu, H.; Qiu, X.; Pidgeon, M.; Dantzig, A. H.; Munroe, J.; Hornback, W. J.; Kasher, J. S.; Glunz, L.; Szczerba, T. *J. Med. Chem.* **1995**, *38,* 590.

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