Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1441–1446

Inhibitors of hepatitis C virus NS3·4A protease 2. Warhead SAR and optimization

Robert B. Perni,* Janos Pitlik, Shawn D. Britt, John J. Court, Lawrence F. Courtney, David D. Deininger, Luc J. Farmer, Cynthia A. Gates, Scott L. Harbeson, Rhonda B. Levin, Chao Lin, Kai Lin, Young-Choon Moon, Yu-Ping Luong, Ethan T. O'Malley, B. Govinda Rao, John A. Thomson, Roger D. Tung, John H. Van Drie and Yunyi Wei

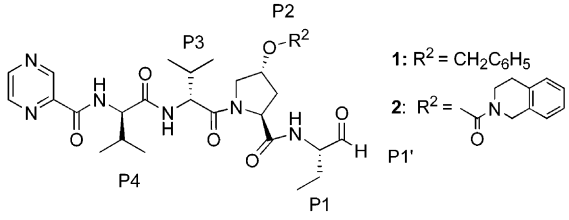
Vertex Pharmaceuticals Inc., 130 Waverly Street, Cambridge, MA 02139, USA

Received 14 October 2003; revised 12 January 2004; accepted 14 January 2004

Abstract—The α-ketoamide warhead (e.g., **15**) was found to be a practical replacement for aliphatic aldehydes in a series of HCV NS3·4A protease inhibitors. Structure–activity relationships and prime side optimization are discussed.

A replacement for the prototype aldehyde covalent warhead was sought for our evolving HCV NS3·4A protease inhibitor series. We describe the SAR of a series of warheads situated on a previously described tetrapeptide scaffold. An unusual α-ketoamide active-site binding motif has been identified and is discussed in detail.

The attractiveness of the NS3·4A protease as a target for inhibiting hepatitis C viral replication has been discussed often over the last several years[1a–b] and the first protease inhibitor, BILN 2061, has recently entered clinical trials.[1c–d] We have recently reported on the structure–activity relationships of a series of tetrapeptide aldehyde inhibitors (e.g., **1**) of the hepatitis C virus NS3·4A protease culminating in the identification of a sub-μM inhibitor, **2** ($K_i = 0.89$ μM).[2] Although useful tools, aliphatic aldehyde warheads are relatively unstable and consequently unlikely to be suitable as drugs. We now describe efforts aimed at replacing aldehyde warheads with functionality that allows for the requisite covalent binding to the catalytic serine while maintaining the necessary chemical stability. The α-ketoamide warhead was found to possess the necessary physicochemical and biochemical characteristics.



It has been established[3] that the preferred P_1 substituent for three of the four natural substrate cleavage sites of the NS3·4A is cysteine.[4] A simple cysteine residue is incompatible with an electrophilic warhead due to intramolecular or intermolecular reactions. We chose aminobutyric (Abu) acid as the initial cysteine replacement providing an ethyl group at P_1. A survey of potential covalent warheads was subsequently performed based on this Abu scaffold. The inhibitory properties of these compounds are summarized in Table 1.

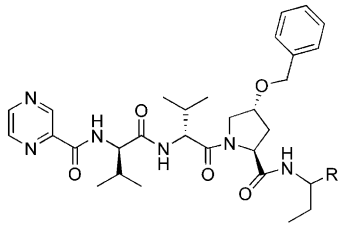
Syntheses of various tetrapeptide inhibitors possessing different electrophilic warheads are described in Schemes 1 and 2.

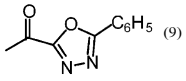
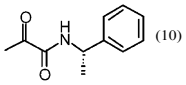
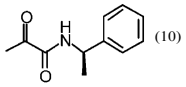
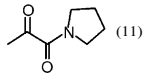
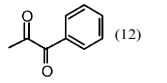
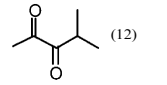
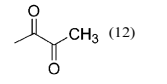
The protected Abu-OH (**3**) was converted to the corresponding 'reduced' warheads using standard methodologies from aldehyde **4**.[5–12] and the resulting reagents (**5a–h**) were used for subsequent coupling reactions (Scheme 2).

Keywords: Hepatitis C; Protease inhibitors.
* Corresponding author. Tel.: +1-617-444-6237; fax: +1-617-444-6766; e-mail: robert_perni@vrtx.com

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.01.022

Table 1. Warhead structure–activity summary



	R[a]	P1 Stereochemistry[b]	K_i (μM, pNA)
1	CHO	L	16
8	COOH	L	> 50
9	$COCF_3$ (5)	D,L	> 50
10	$COCH_2Cl$ (6)	D,L	> 50
11	$COCF_2CO_2C_2H_5$ (7)	D,L	> 50
12	$COCF_2CF_3$ (8)	D,L	17.5
13	 (9)	L	35
14	$COCONHCH_2C_6H_5$ (10)	L	4.0
15	 (10)	L	0.92
16	 (10)	L	17
17	 (11)	L	32
18	 (12)	L	10
19	 (12)	L	2.7
20	 (12)	L	50

[a] References for synthesis of warheads are provided in parentheses.
[b] Where a mixture is denoted (**9**–**12**) both isomers were obtained in the preparation of the warheads and carried through to final product. The L form of **1** was obtained by chromatographic separation of P_1 epimers. All other compounds were prepared as single isomers.

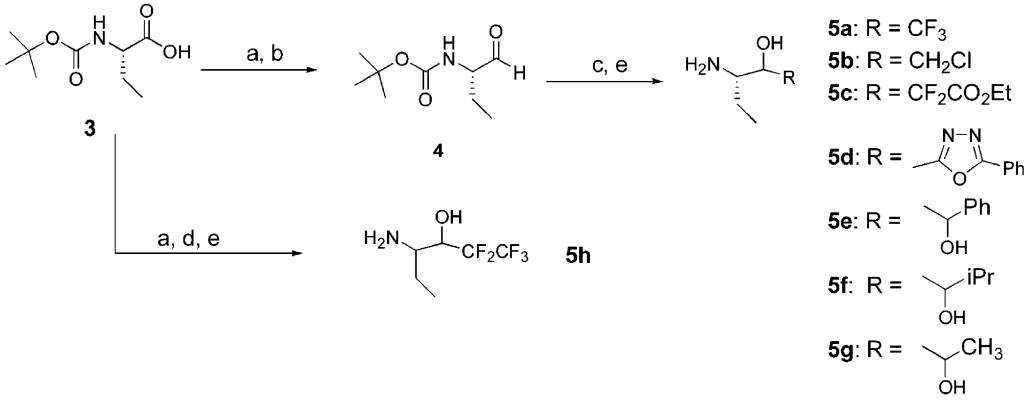
Due to the commercial availability of 4-OBn-hydroxyproline, compound **6** was used as a standard scaffold for our warhead exploration and was synthesized using previously described methodology.[2] Carboxylic acid **6** was coupled with H-Abu-O*t*Bu under normal peptide coupling conditions. The *t*-butyl protecting group of the resulting tetrapeptide was subsequently cleaved under acidic conditions to provide the free acid, **8**. Alternatively, **6** was coupled with warhead precursors **5a**–**h** resulting in formation of hydroxy derivatives **7a**–**h**. The resulting compounds were oxidized to the corresponding ketones **9**–**13**, and **18**–**20** by using Dess–Martin periodinate, Swern, or Moffatt–Pfitzner oxidation systems. The oxidant was chosen based on the compatibility of the R groups with the reagents. Compounds **14**–**17** were prepared as previously described.[12]

Ketoamide variants were prepared by coupling **21**[2] to solid supported hydroxyester **22**, which was prepared analogously to the warheads in Scheme 1 and subsequently coupled to the THP-resin.[13] Conversion of **23** to the desired amides was carried out under standard conditions to give **24**, **30** and **32**–**50**. The protected carboxylates, **45**–**50**, were finally converted to free acids with TFA to give **25**–**29** and **31** (Scheme 3).

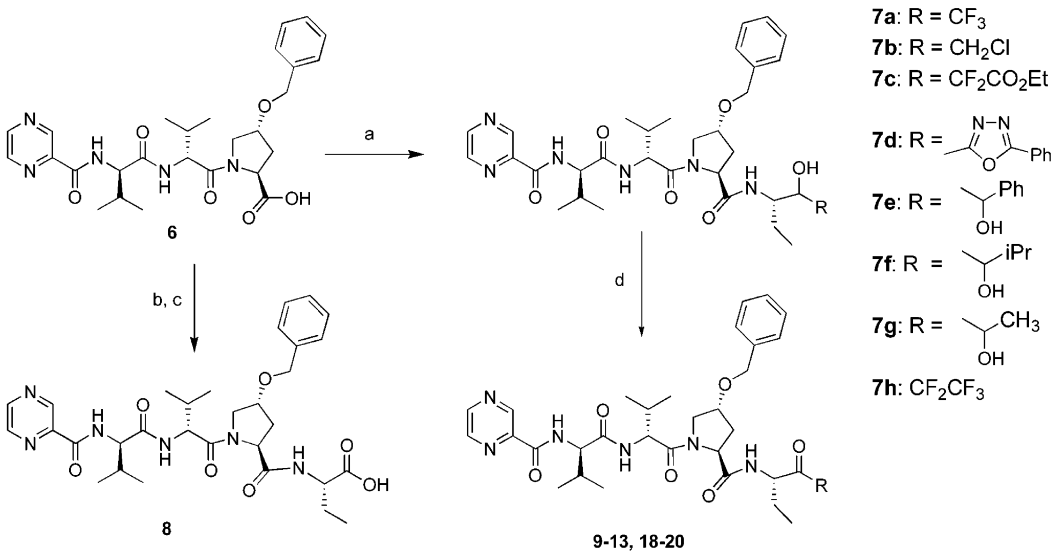
Compounds were initially tested using a spectrophotometric assay described by Landro et. al.[14] As the potency of the compounds increased and exhibited tight binding inhibition, the limits of accuracy for the p-nitroaniline assay were exceeded. This is due to the relatively high concentration of protease used in the spectrophotometric assay (200–500 nM). Compounds with K_i values less than 200 nM were thus evaluated using a reverse phase HPLC based assay in which substantially lower enzyme concentrations may be used.

A non-covalent, charged warhead theoretically could provide binding efficiency via electrostatic interactions without requiring formation of a covalent bond. Carboxylate **8**, however failed to show any advantage over **1**. Given the lack of significant binding affinity and potential problems associated with charged groups (e.g., inhibition of cellular penetration) we did not pursue this class of inhibitors further. Somewhat surprisingly, known covalent warheads that have been widely used as serine and cysteine protease inhibitors, such as haloketones and heterocyclic ketones (**9**–**13**), failed to show improved binding relative to aldehydes. The results for α-haloketone warheads are consistent with those previously published for non-covalent hexapeptide inhibitors.[15] Certain α-ketoamide warheads, however, did demonstrate improved binding characteristics.[10a,16] A benzylamide **14**, for example, was four time more potent than **1**. The addition of a branching methyl group at the benzylic carbon of **14** in the S-configuration afforded **15**. Exhibiting a K_i value of 0.92 μM, **15** represents a substantial improvement over **1**. A tertiary ketoamide, **17**, was less active than **15**. The 1-(*R*)-1-phenylethyl derivative **16** possessing opposite stereochemistry from 1-(*S*)-1-phenylethyl analogue **15** was also less active than **15**. This stereochemical preference has been previously observed.[17] α-Diketone **19** displayed slightly better potency relative to **1** while diketones **18** and **20** did not. Based on this data α-ketoamides were chosen as the warhead on which to perform prime-side optimization.

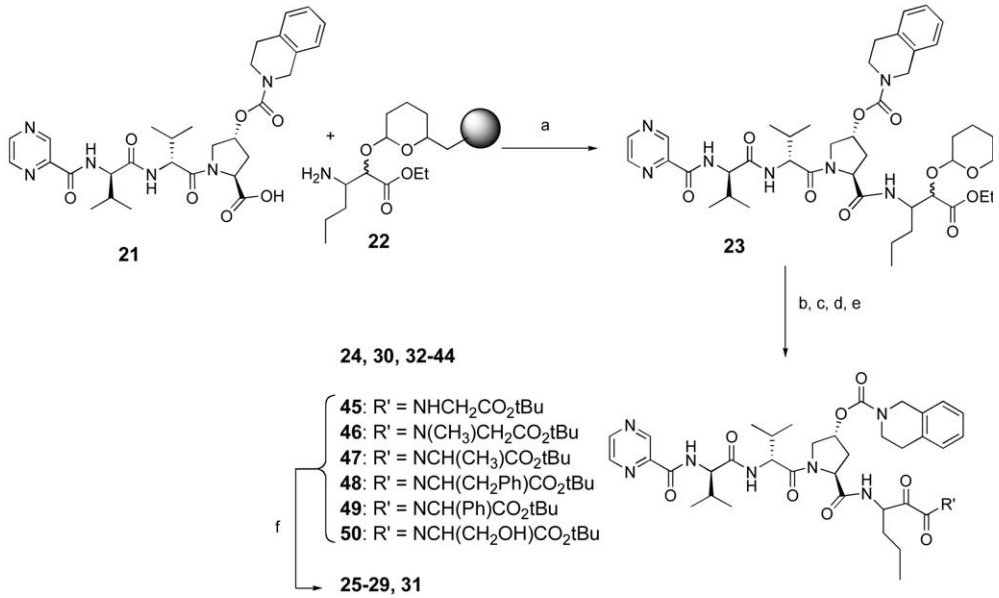
Analysis of the X-ray structure of the bound inhibitor/tNS3·4A complex of **15** at least partially, explains the excellent binding properties of molecules containing α-ketoamide warheads (Fig. 1). The 'normal' orientation of tetrahedral intermediates being cleaved by the NS3·4A enzyme provides for the carbonyl oxygen of the intermediate to be stabilized by the NH's located in the so-called oxyanion hole (defined by the Ser139 and Gly137).[18] This arrangement does not occur for ketoamides bound to the HCV NS3·4A protease. In this case the keto–oxygen points toward solvent while the amide carbonyl is oriented and hydrogen bonded to the oxyanion hole N–H's. A related bidentate type of motif



Scheme 1. (a) $HN(CH_3)OCH_3$, CDI, DIEA; (b) $LiAlH_4$; (c) See refs 5–11; (d) CF_3CF_2I, CH_3Li, LiBr; (e) TFA. CH_2Cl_2.



Scheme 2. (a) **5a–h** PyBrop, HOBt, DIEA, DMF; (b) H-Abu-O*t*Bu, PyBrop, DIEA, DMF; (c) TFA, CH_2Cl_2; (d) Oxidation.



Scheme 3. (a) PyBrop, HOBt, DIEA, DMF; (b) KOTMS, THF; (c) $R'NH_2$ or $HN(CH_3)CH_2CO_2tBu$, PyBrop, HOBt, DIEA, DMF; (d) pTsOH, CH_3OH or PPTS, CH_2Cl_2, *n*BuOH; (e) Oxidation; (f) 50% TFA/CH_2Cl_2.

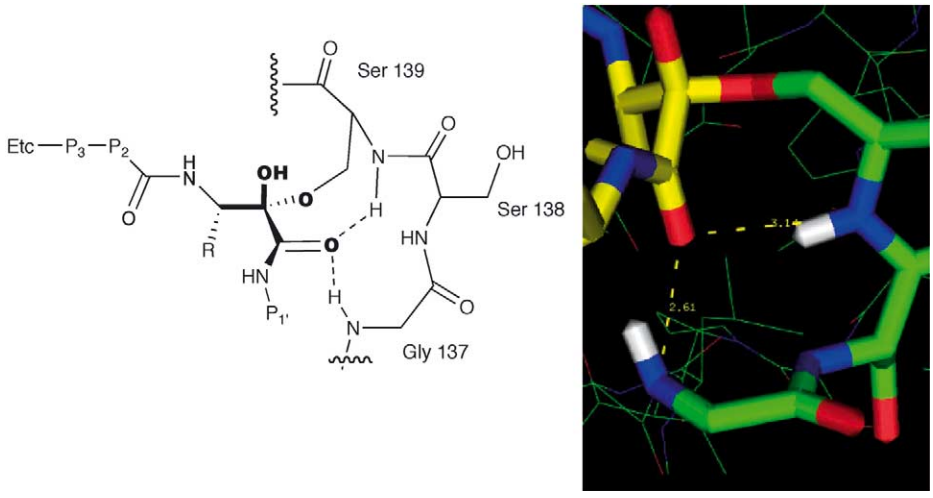


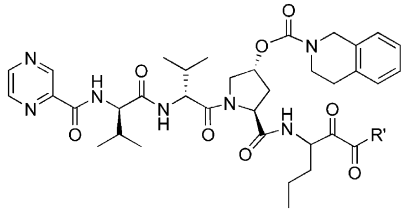
Figure 1. Schematic (left) of ketoamide binding motif and an X-ray structure (right) of the warhead of **15** bound to the NS3·4A enzyme active site. Inhibitor shown in yellow and protein shown in green. The resolution of this structure is 2.9 Å.

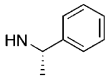
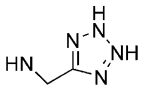
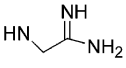
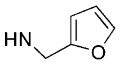
has been described previously for α-ketoacids bound to the NS3·4A protease.[19] The keto–acid moiety generates additional binding energy via electrostatic interaction of the charged carboxylate and the protein. For keto–acid warheads interacting with the NS3·4A kinetic data implicates that in addition to the stabilization of a free terminal carboxylate by Gly137, the catalytic His57 also participates in stabilizing the complex.[18] The X-ray structure shown in Figure 1 indicates that such an electrostatic interaction is not required for efficient binding of inhibitors such as **15**.

A series of α-ketoamide based inhibitors derived from **2**[2] were prepared where the amide nitrogen substitution was varied. The inhibitory data is summarized in Table 2. Both spectrophotometrically and HPLC derived data are provided for the more potent examples. Prime-side termini comprised of free amino acids possess the most potent inhibitory activity probably due to non-specific electrostatic attraction of the terminal carboxylate with the preponderance of arginines in the prime-side binding groove. Charged **25**, **28**, **29**, are more potent than the methylbenzyl derivative, **24** while **26** and **27** were significantly less potent. Inhibitory constants for **28** and **29** are 0.13 μM and 0.026 μM resepctively, whereas the phenylalanine methyl ester derivative, **30**, is approximately 20-fold less potent than **29**. Acid replacements such as methyltetrazole, **34**, result in good but unexceptional activity. Simple polar groups such as cyano provide less binding potency. The same may be said for a furanyl derivative, **36**. Surprisingly, small aliphatic groups, compounds **39**–**41**, exhibit good potency. A *t*-butyl derivative is not well accommodated at this position and therefore the activity of **42** is significantly reduced relative to the other aliphatic examples. A *N*-methylglycine derivative, **26**, related to **25** is >100-fold less potent than **25**. An azirdine derived ketomaide **32**, is slightly more potent than **26** as is azetidine **43**.

Cellular activity was determined in an HCV replicon assay, where the replicon cells[20] were treated with the

Table 2. α-Ketoamide structure–activity summary



	R′[a]	K_i μM (pNA)	K_i μM (HPLC)
24		<0.2 (0.10)	0.22
25	Gly	<0.2 (0.15)	0.037
26	$N(CH_3)CH_2CO_2H$	5.23	—
27	Phenylgly	1.63	—
28	Ala	<0.2 (0.18)	0.13
29	Phe	<0.2 (0.03)	0.026
30	Phe methyl ester	0.43	—
31	Ser	0.54	—
32		1.6	—
33	$NHCH_2CN$	1.12	—
34		0.29	—
35		5.76	—
36		4.72	—
37	Naphthylalanine	<0.2 (0.18)	0.75
38	$NHCH_2C_6H_5$	<0.2 (0.15)	0.22
39	NH-Cyclopropyl	0.22	0.42
40	NH-Isopropyl	0.52	—
41	NH-Cyclobutyl	0.80	—
42	NH-*t*-butyl	6.90	—
43		0.97	—
44	$NHCH_2CH_2C_6H_5$	0.21	0.52

[a] Compounds were prepared as mixtures of P_1 diastereomers.

Table 3. Replicon results (Huh 7 cells)

	IC_{50} (μM)	CC_{50} (μM)
15	4.8	>100
24	0.31	16
25	>10	>100
29	>10	>100
30	>10	42
34	>10	>100
38	3.2	>30
39	2.3	>100
40	>10	>100
41	10.2	>100
43	10.3	>100
44	>10	20

compound for 48 h, then the reduction of HCV RNA in the cells (IC_{50}) was determined by quantitative RT-PCR and the cytotoxicity of the compound (CC_{50}) was determined by MTS-based cell viability assay. These data are presented in Table 3. The charged inhibitors, **25** and **29**, exhibit no inhibitory effects at concentrations as high as 10 μM potentially due to lack of cellular penetration. The methyl ester **30**, still showed no significant activity but cytotoxicity was increased. Although BILN-2061[1c] contains a free carboxylate and has recently demontrated clinical proof-of-concept, free carboxylates appear to preclude cellular activity in our series. Conversely, neutral analogues such as **15**, **38** and **39**, show good activity in the replicon assay. The P_2 optimized analogue of **15**, compound **24**, displayed improved cellular potency relative to **15**. Azetidine **43**, which possesses moderate enzymatic activity, exhibits an $IC_{50}=10.3$ μM, almost identical to that of the cyclobutyl derivative, **41** ($IC_{50}=10.2$ μM). An acid isostere analogue, **34**, did not show activity below 10 μM. Similarly, **40** and **44**, containing isopropyl and phenethyl $P_{1'}$ termini respectively, did not show significant activity at 10 μM concentrations.

The $P_{1'}$ optimzation exercize teaches that a wide variety of sizes and polarities are tolerated but that predictions based on this SAR are difficult. Larger groups can extend along the active site binding groove and smaller groups, even if uncharged, can be folded, at least to a degree, into the protein surface because of plasticity of the NS3 surface proteins.[1] While an acidic prime-side terminus is optimal for enzymatic binding these groups appear to preclude cellular activity presumably because of poor cellular penetration. Small, neutral α-ketoamide warheads were found to possess the necesssary physicochemical and biochemical characteristics to show inhibitory activity in the HCV replicon assay. Based on these results this series of inhibitors has the potential to evolve into effective therapeutic agents against hepatitis C infection. Optimization efforts in this series continue and results will be reported in the near future.

References and notes

1. (a) Narjes, F.; Koch, U.; Steinkuhler, C. *Exp. Op. Inves. New Drugs* **2003**, *12*, 153. (b) Perni, R. B.; Kwong, A. D. In *Progress in Medicinal Chemistry*, King F. D. and Oxford, A.W. (Eds.) Elsevier Science B.V., 2002, p 215. (c) Lamarre, D.; Bailey, M.; Bolger, G.; Cameron, D.; Cartier, M.; Faucher, A.-M.; Goudreau, N.; Kukolj, G.; Lagace, L.; Pause, A.; Rancourt, J.; Thibeault, D.; Tsantrizos, Y.; Llinas-Brunet, M. *Hepatology* **2002**, *36*, 92A (Abstract #464). (d) Hinrichsen, H.; Benhamou, Y.; Reiser, M.; Sentjens, R.; Wedemeyer, H.; Calleja, J. L.; Forns, X.; Cronlein, J.; Nehmiz, G.; Steinmann, G. *Hepatology* **2002**, *36*, 379A (Abstract #866).
2. Perni, R. B.; Britt, S. D.; Court, J. J.; Courtney, L. F.; Deininger, D. D.; Farmer, L. J.; Gates, C. A.; Harbeson, S. L.; Kim, J. L.; Landro, J. A.; Levin, R.; Luong, Y.-P.; O'Malley, E. T.; Pitlik, J.; Rao, G.; Thomson, J. A.; Tung, R. D.; Van; Drie, J. H.; Wei, Y. *Bioorg. Med Chem. Lett*. **2003**, *13*, 4059.
3. Urbani, A.; Bianchi, E.; Narjes, F.; Tramontano, A.; De Francesco, R.; Steinkühler, C.; Pessi, A. *J. Biol. Chem.* **1997**, *272*, 9204.
4. For a description of protease sub-site nomenclature see: Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
5. (a) Edwards, P. D. *Tetrahedron Lett*. **1992**, *33*, 4279. (b) Peet, N. P.; Burkhart, J. P.; Angelastro, M. R.; Giroux, E. L.; Mehdi, S.; Bey, P.; Kolb, M.; Neises, B.; Schirlin, D. *J. Med. Chem*. **1990**, *33*, 394.
6. (a) Getman, D. P.; DeCrescenzo, G. A.; Heintz, R. M.; Reed, K. L.; Talley, J. J.; Bryant, M. L.; Clare, M.; Houseman, K. A.; Marr, J. J.; Mueller, R. A.; Vazquez, M. L.; Shieh, H.-S.; Stallings, W. C.; Stegeman, R. A. *J. Med. Chem*. **1993**, *36*, 288. (b) Concellon, L. M.; Riego, E.; Rodriguez-Solla, H.; Plutin, A. M. *J. Org. Chem.* **2001**, *66*, 8661.
7. (a) Lang, R. W.; Schaub, B. *Tetrahedron Lett*. **1988**, *29*, 2943. (b) Ref. 5b.
8. (a) Angelastro, M. R.; Burkhart, J. P.; Bey, P.; Peet, N. P. *Tetrahedron Lett*. **1992**, *33*, 3265. (b) Angelastro, M. R.; Baugh, L. E.; Bey, P.; Burkhart, J. P.; Chen, T.-M.; Durham, S. L.; Hare, M. C.; Huber, E. W.; Janusz, M. J.; Koehl, J. R.; Marquart, A. L. *J. Med. Chem*. **1994**, *37*, 4538.
9. Ohtomo, K.; Yamamoto, T.; Okuma, M.; Horiuchi, T.; Imanishi, H.; Odagaki, Y.; Kawabata, K.; Sekioka, T.; Hirota, Y.; Matsuoka, S.; Nakai, H.; Toda, M. *J. Med. Chem*. **2001**, *44*, 1268.
10. (a) Tung, R. D.; Harbeson, S. L.; Deininger, D. D.; Murcko, M. A.; Bhisetti, G. R.; Farmer, L. J. US 62653880, **2001**, PCT WO 98/17679, 1998. (b) Harbeson, S. L.; Abelleira, S. M.; Akiyama, A.; Barrett, R.; Carroll, R. M.; Straub, J. A.; Tkacz, J. N.; Wu, C.; Musso, G. F. *J. Med. Chem*. **1994**, *37*, 2918. (c) Banfi, L.; Guanti, G.; Riva, R.; Basso, A.; Calcagno, E. *Tetrahedron Lett*. **2002**, *43*, 4067.
11. Perni, R.; Court, J.; O'Malley, E.; Rao, B.G. PCT WO 0174768 **2001**.
12. Farmer, L.; Pitlik, J.; Perni, R.; Courtney, L.; Van Drie, J. PCT WO 03006490 2003.
13. Kick, E. K.; Ellman, J. A. *J. Med. Chem*. **1995**, *38*, 1427.
14. HPLC Protocol The HPLC protocol used was a modification of that described in Landro et al. (ref 14b). Enzyme activity was measured by separation of substrate and products using a reversed phase HPLC microbore column (Phenomenex Jupiter C18, 5 μm, 150×2 mm; column heated to 40C; 5%–60% acetonitrile with 0.1% trifluoroacetic acid as the counterion at 4.6% per min) and a Hewlett-Packard 1100 system with autoinjection, thermostatted column chamber, and a diode array detection at 210 and 280 nm. K_i values were calculated from rate versus inhibitor concentration by non-linear least square fitting to the tight binding inhibition equation (Ref. 13a)

using Prism software (GraphPad) (a) Morrison, J. F. *Biochim. Biophys Acta* **1969**, *185*, 269. (b) Landro, J. A.; Raybuck, S. A.; Luong, Y. P. C.; O'Malley, E. T.; Harbeson, S. L.; Morgenstern, K. A.; Rao, G.; Livingston, D. J. *Biochemistry* **1997**, *36*, 9340.
15. Llinas-Brunet, M.; Bailey, M.; Deziel, R.; Fazal, G.; Gorys, V.; Goulet, S.; Halmos, T.; Maurice, R.; Poirier, M.; Poupart, M.-A.; Rancourt, J.; Thibeault, D.; Wernic, D.; Lamarre, D. *Biorg. Med. Chem. Lett.* **1998**, *8*, 2719.
16. Han, W.; Hu, Z.; Jiang, X.; Wasserman, Z. R.; Decicco, C. P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1111.
17. Bennett, J. M.; Campbell, A. D.; Campbell, A. J.; Carr, M. G.; Dunsdon, R. M.; Greening, J. R.; Hurst, D. N.; Jennings, N. S.; Jones, P. S.; Jordon, S.; Kay, P. B.; O'Brien, M. A.; King-Underwood, J.; Raynham, T. M.; Wilkinson, C. S.; Wilkinson, T. C. I.; Wilson, F. X. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 355.
18. Narjes, F.; Brunetti, M.; Colarusso, S.; Gerlach, B.; Koch, E.; Biasol, G.; Fattori, D.; De Francesco, R.; Matassa, V. G.; Steinkühler, C. *Biochemistry* **2000**, *39*, 1849.
19. Di Marco, S.; Rizzi, M.; Volpari, C.; Walsh, M. A.; Narjes, F.; Colarusso, S.; De Francesco, R.; Matassa, V. G.; Sollazzo, M. *J. Biol. Chem.* **2000**, *275*, 7152.
20. Lohmann, V.; Korner, F.; Koch, J.-O.; Herian, U.; Theilmann, L.; Bartenschlager, R. *Science* **1999**, *285*, 110.