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Highly Potent Non-peptidic Inhibitors of the HCV NS3/NS4A Serine Protease

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Abstract—Screening of a diverse set of bisbenzimidazoles for inhibition of the hepatitis C virus (HCV) serine protease NS3/NS4A led to the identification of a potent Zn^{2+}-dependent inhibitor (**1**). Optimization of this screening hit afforded a 10-fold more potent inhibitor (**46**) under Zn^{2+} conditions ($K_i = 27$ nM). This compound (**46**) binds also to NS3/NS4A in a Zn^{2+} independent fashion ($K_i = 1$ μM). The SAR of this class of compounds under Zn^{2+} conditions is highly divergent compared to the SAR in the absence of Zn^{2+}, suggesting two distinct binding modes.

The prevalence of chronic Hepatitis C virus (HCV) infections in the US was recently determined to be around 1.8% (2.7 million people), with about 15,000 new cases every year.[1] Worldwide, HCV is estimated to infect 170 million people.[1] Over 85% of all cases become chronic.[2,3] Current therapies of HCV infections with interferons show only modest results and have serious side effects such as depression, fever, and fatigue. Recently, combination treatment with ribavarin,[4] a broad spectrum antiviral and pegylated interferon[5] has proven to be superior to monotherapy. Despite these advances, a broadly effective antiviral therapy for HCV is still elusive. An excellent review by Dymock summarizes emerging therapies for HCV.[6]

Significant research efforts are currently directed towards targeting viral enzymes. The HCV RNA genome is translated into a 9.5 kb polyprotein (C-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B), which is then processed into the active viral proteins. Host cell proteases are responsible for cleavages in the C-E1-E2-P7-NS2 region.[7] The NS2-NS3 cleavage is performed by NS2, most likely a Zn^{2+} containing metalloprotease.[8] The remaining processings of the NS3-NS4A-NS4B-NS5A-NS5B fragment are all dependent on the NS3/NS4A protease, which makes this chymotrypsin-like serine protease a promising target.[7] Highly potent substrate-based peptidic inhibitors for the HCV NS3/NS4A protease were developed (Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH, $K_i = 1.5$ nM).[9] Non peptidic small molecule inhibitors for NS3/NS4A were published by Rational Drug Design Laboratories[10] and Schering-Plough[11] with activities in the low micromolar range.

We recently published a new class of highly potent, reversible and selective serine protease inhibitors.[12] The binding of these new inhibitors, which are derived from a bis-benzimidazolemethane fragment, is dependent on the presence of Zn^{2+}. X-ray structure determination revealed a Zn^{2+} ion at the catalytic site, which is coordinated by His 57 and Ser 195 of the enzyme, and by two imidazole-nitrogens of the bidentate ligand bis-benzimidazole.[12] We explored this binding motif for the

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inhibition of the HCV NS3/NS4A protease and screened a library of a diverse set of bis-benzimidazoles. Indeed, bis-benzimidazole **1** [CRA-6336 (APC-6336), Fig. 1] showed an activity of 0.20 μM in the presence of Zn^{2+} (12.5 μM).[13] Sequestering of Zn^{2+} with excess ethylenediamintetraacetate (EDTA, 25 μM) induced a drop in binding affinity of about 800-fold. In contrast, the presence of Zn^{2+} increased the affinity of peptidic inhibitors for HCV NS3/NS4A only by about 2-fold. Kinetic studies showed that **1** is a competitive, active site directed inhibitor of NS3/NS4A (data not shown). Screening hit **1** is over 100-fold selective for HCV NS3/NS4A compared to the serine proteases chymotrypsin, tryspsin and elastase. We have systematically explored the structure–activity relationship (SAR) of screening hit **1** and were able to identify bis-benzimidazoles with improved binding affinity in the presence and absence of Zn^{2+}.

Studies to determine the key binding elements in screening hit **1** are summarized in Figures 1–3. Masking of one negative charge of the phosphonic acid moiety [–PO_3H_2→–PO_2(OMe)OH] results in a loss of binding affinity in the presence of Zn^{2+} of almost one order of magnitude. Surprisingly, the binding affinity in the presence of EDTA improved more than 4 times. Complete deletion of the phosphonic acid as in the case of glycine **3** abolishes most of the activity under Zn^{2+} conditions and all activity under EDTA conditions. Enantiopure **1** derived from either (*R*)- or (*S*)-phosphonoalanine did not show a significant difference in binding in the presence or absence of Zn^{2+} (data not shown).

The orientation of the phosphonoalanine side chain relative to the bis-benzimidazole scaffold appears to be crucial for binding (see Fig. 2). Moving the phosphonoalanine side chain from the 6-position in benzimidazole **5**, to the 5-position (**4**), or to the 7 position (**6**) abolishes most of the

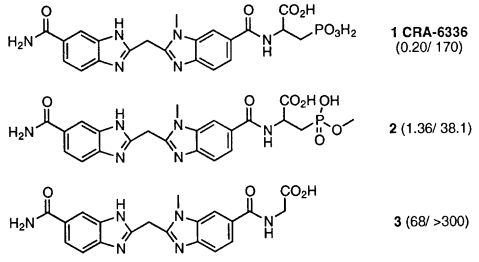


Figure 1. Screening hit **1** and analogues **2** and **3**. In parentheses, K_i (μM) for inhibition of HCV NS3/NS4A in the presence of Zn^{2+}/EDTA.[13]

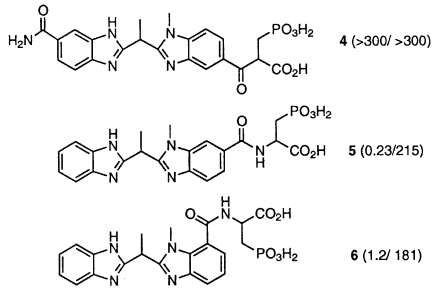


Figure 2. Phosphonoalanine analogues of **5**. In parentheses, K_i (μM) for inhibition of HCV NS3/NS4A in the presence of Zn^{2+}/EDTA.[13]

binding. The phosphonoalanine side chain SAR of **1** was explored further in detail and resulted in the identification of a novel series of active compounds under Zn^{2+} conditions in the low micromolar range.[14]

The observation that the binding affinity of **1** shifts three orders of magnitude in the presence or absence of Zn^{2+}, suggests a Zn^{2+} binding motif as reported.[12,15] X-ray structure determination revealed this type of binding motif in similar inhibitor–enzyme complexes of trypsin and thrombin. Gradual deletion of the bisbenzimidazole moiety in these inhibitor–enzyme complexes resulted in a gradual loss of binding to thrombin or trypsin. We have seen similar SAR trends in **1** (see compounds **7**–**9**, Fig. 3), which also support a binding mode as reported.[12] However, in the absence of structural information, other zinc-mediated binding modes may also be available to these inhibitors. The SAR shown in Figures 1–3 suggests that the key binding element of **1** involves a Zn^{2+}-mediated chelation of the benzimidazole scaffold and an electrostatic interaction of the acidic phosphonalanine residues with NS3/NS4A. Deletion of either binding element results in complete loss of activity.

To further improve the activity, we prepared a library of analogues of **1** which have different electronic properties and steric demand at the left-hand benzimidazole moiety. The above-mentioned key binding elements were retained (see Table 1). Overall, the SAR under Zn^{2+} conditions is unresponsive. Compared to parent compound **1** (see also **35**), electron withdrawing substituents (**10**–**18**) and electron donating substituents (**19**–**23**) did not produce more active leads. A methyl group seems not to be tolerated in the R_4 position (**20**). Similarly, in the bridgehead monofluoro series (**24**–**26**), a relatively flat SAR under Zn^{2+} conditions was observed. Bridgehead difluorination (**27**–**33**) appears to reduce the activity under Zn^{2+} as well as under EDTA conditions. Bridgehead difluoro-bisbenzimidazole **27** had an exceptionally high activity of 1.1 μM under EDTA conditions. A survey of the *N*-alkyl substituent of the right-hand benzimidazole also revealed a flat SAR (**35**–**40**).

Studies by Pessi et al.[9] of peptide-based inhibitors of HCV NS3/NS4A revealed that charge–charge interaction at P_6 and P_5 in conjunction with a carboxylic acid at the C-terminus is crucial for binding. We wondered if this binding interaction could be translated to the bis-benzimidazole scaffold. Indeed, installation of an additional phosphonoalanine side chain at the left hand side resulted in a significant increase in binding both under Zn^{2+} and EDTA conditions (**41**, see Fig. 4).

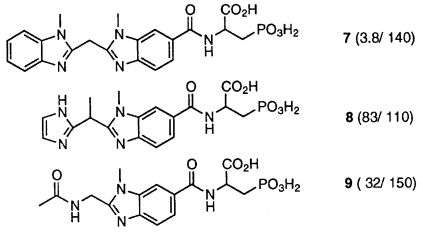
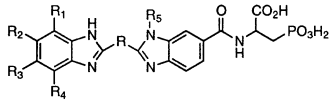


Figure 3. Left-hand deletion SAR. In parentheses, K_i (μM) for inhibition of HCV NS3/NS4A in the presence of Zn^{2+}/EDTA.[13]

Table 1. SAR of the bis-benzimidazole scaffold. K_i (μM) for inhibition of HCV NS3/NS4A in the presence of Zn^{2+} or EDTA[13]



Compd	R	R_1	R_2	R_3	R_4	R_5	K_i (Zn^{2+})	K_i (EDTA)
10	$-CH_2-$	H	H	F	H	CH_3	0.28	95
11	$-CH_2-$	H	H	Cl	H	CH_3	0.38	130
12	$-CH_2-$	H	H	CF_3	H	CH_3	0.41	70
13	$-CH_2-$	H	F	F	H	CH_3	0.21	25
14	$-CH_2-$	H	F	H	F	CH_3	0.24	58
15	$-CH_2-$	H	H	F	F	CH_3	0.27	140
16	$-CH_2-$	F	F	F	H	CH_3	0.2	21
17	$-CH_2-$	F	F	H	F	CH_3	0.35	22
18	$-CH_2-$	F	F	F	F	CH_3	0.28	17
19	$-CH_2-$	H	H	Me	H	CH_3	0.24	98
20	$-CH_2-$	H	H	H	Me	CH_3	10	>300
21	$-CH_2-$	H	H	OH	H	CH_3	0.19	250
22	$-CH_2-$	H	H	OMe	H	CH_3	0.2	187
23	$-CH_2-$	H	H	H	OMe	CH_3	0.49	24
24	–CHF–	H	H	H	H	CH_3	0.47	37
25	–CHF–	F	F	H	F	CH_3	0.55	72
26	–CHF–	H	F	H	F	CH_3	0.66	91
27	$-CF_2-$	H	H	H	H	CH_3	0.25	1.1
28	$-CF_2-$	H	$CONH_2$	H	H	CH_3	1.2	74
29	$-CF_2-$	H	H	F	H	CH_3	1.3	55
30	$-CF_2-$	H	H	Cl	H	CH_3	2.4	38
31	$-CF_2-$	H	F	F	H	CH_3	4	15
32	$-CF_2-$	H	F	H	F	CH_3	1.4	25
33	$-CF_2-$	F	F	H	F	CH_3	2.9	12
34	$-CF_2-$	H	H	H	Me	CH_3	21	66
35	$-CH_2-$	H	H	H	H	CH_3	0.45	>300
36	$-CH_2-$	H	H	H	H	*i*-Pr	12	>300
37	$-CH_2-$	H	H	H	H	*cyclo*-PrCH_2	0.14	140
38	$-CH_2-$	H	H	H	H	*i*-Bu	0.37	>300
39	$-CH_2-$	H	H	H	H	*neo*-Pentyl	0.61	>300
40	$-CH_2-$	H	H	H	H	*i*-Pentyl	0.87	>300

Bridgehead difluorination (**42**) improves the activity to 73 nM under Zn^{2+} conditions and 15 μM under EDTA conditions. While masking of the negative charge in the carboxylic acid moieties as in **43** did not have a significant effect, the introduction of benzyl esters as in **44** completely impairs binding to NS3/NS4A. This observation is in line with the low activities of **2** and **3**, which lack the negative charges in the phosphonic acid moieties.

Further elaboration of the right-hand phosphonoalanine side chain with another phosphonoalanine as in (**45**) (see Fig. 5) to mimic the P_5/P_6 interaction improved the binding to NS3/NS4A only under Zn^{2+} conditions. However, in conjunction with a third phosphonoalanine moiety at the right-hand position as in compound (**46**), the binding to NS3/NS4A improved to 27 nM under Zn^{2+} conditions and 1 μM under EDTA conditions.

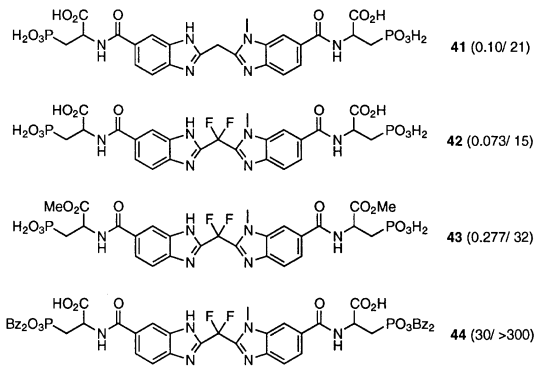


Figure 4. Bis-phosphonate analogues of **1**. In parentheses, K_i (μM) for inhibition of HCV NS3/NS4A in the presence of Zn^{2+}/ EDTA.[13]

While trends in the SAR under EDTA conditions leading to **46** and trends in the SAR of peptidic inhibitors are similar, we do not have evidence for similar binding modes of **46** and peptidic inhibitors.

The synthesis of **46** is summarized below: Esterification of *N*-Boc serine followed by conversion to the dehydroalanine and reaction with $P(OH)(OBn)_2$ in the presence of DBU gave orthogonally protected phosphonoalanine (Scheme 1). Ester cleavage afforded

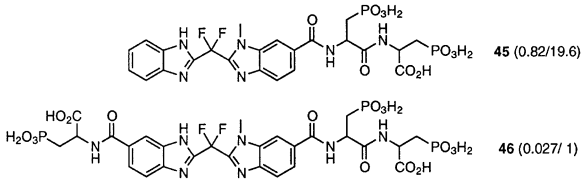
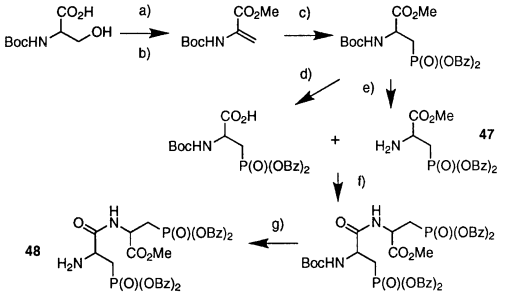
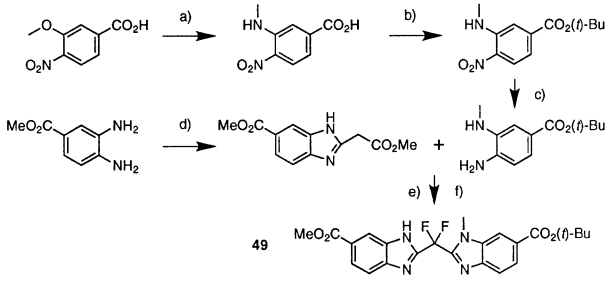


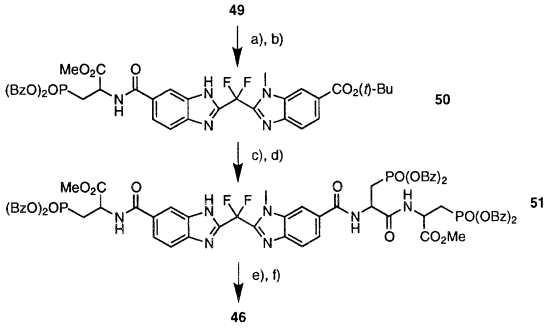
Figure 5. Bis- and tris-phosphonoalanine analogues of **27** (mixtures of diastereoisomers). In parentheses, K_i (μM) for inhibition of HCV NS3/NS4A in the presence of Zn^{2+} or EDTA.[13]



Scheme 1. Synthesis of **47** and **48**: (a) $TMSCH_2N_2$, MeOH/benzene, 95%; (b) PPh_3, DEAD, toluene, rt, 85%; (c) $P(OH)(OBn)_2$, DBU, neat, 65%; (d) LiOH, THF, H_2O, 80%; (e) TFA, CH_2Cl_2, 68%; (f) PyBop, DMF, NMM, 93%; (g) TFA, CH_2Cl_2, 95%.



Scheme 2. Synthesis of **49**: (a) 40% $MeNH_2$ in H_2O, 100 °C, 82%; (b) $(t\text{-BuO})_2CHNMe_2$, toluene, 80 °C, 78%; (c) H_2, Pd/C, MeOH/HOAc, 87%; (d) $MeO(CN)CH_2CO_2Me$, EtOH, 80 °C, 77%; (e) neat, 150 °C, 63%; (f) $(PhSO_2)_2NF$, MeOH, 70 °C, 80%.



Scheme 3. Synthesis of **46**: (a) LiOH, MeOH/H_2O, 90 °C, 60%; (b) **47**, PyBop, DIPEA, DMF, rt, 62%; (c) TFA, rt, 89%; (d) **48**, PyBop, DIPEA, DMF, rt, 79%; (e) LiOH, MeOH/H_2O, 61%; (f) Pd/C, MeOH/HCl, 10%.

the acid **47**, which was coupled into the *N*-deprotected phosphonoalanine to give **48** after TFA cleavage.

Scaffold **49** was prepared via a thermal cyclocondensation at 150 °C of a phenylendiamine and the right-hand benzimidazole acetic acid methyl ester (Scheme 2). Bridgehead fluorination was accomplished by heating with *N*-fluoro-bisphenylsulphonimide in MeOH.

Methyl ester cleavage of **49** followed by coupling with **47** afforded the left-hand side phosphonoalanine (Scheme 3). TFA cleavage of the right hand ester and coupling with **48** gave fully protected **46**. Deprotection to **46** was accomplished by cleavage of the methyl esters, followed by global benzyl ester cleavage using Pd/C. All compounds for assay were purified by reverse-phase HPLC (H_2O/MeCN/0.1% TFA).

In summary, the binding affinity of screening hit **1** under Zn^{2+} conditions was improved by the introduction of a phosphonoalanine side chain on the left-hand side in conjunction with elaboration of the right-hand phosphonoalanine to a bisphosphonate **46**. It was found that the specific display of negative charges presented by the phosphonic acid moieties is responsible for most of the binding in the side chain. Attempts to replace the phosphonic acid moieties by either less-charged groups or by groups which can be masked by a prodrug approach were reported[14] and resulted in only moderately active compounds. Sequestering of Zn^{2+} with EDTA results in a drop of activity in **46** from 27 nM to 1 μM. Divergent trends in the SAR were found under EDTA conditions compared to Zn^{2+} conditions. This implies two different binding modes. Both binding modes depend heavily on the polyanionic character of this class of inhibitors, which is reminiscent of the SAR of substrate based peptidic inhibitors of NS3/NS4A.[9,16–18]

Acknowledgements

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References and Notes

1. Cohen, C. *Science* **1999**, *285*, 26.
2. Cuthbert, J. *Clin. Microbiol. Rev.* **1994**, *7*, 505.
3. El-Serag, H.; Mason, A. *Arch. Int. Med.* **2000**, *160*, 3227.
4. Mutchnick, M. G.; Lindsay, K. L.; Schiff, E. R.; Cummings, G. D.; Appelman, H. D.; Peleman, R. R.; Silva, M.; Roach, K. C.; Simmons, F.; Milstein, S.; Gordon, S. C.; Ehrinpreis, M. N. *N. Engl. J. Med.* **1998**, *339*, 1493.
5. Heathcote, E. J.; Shiffman, M. L.; Cooksley, W. G.; Dusheiko, G. M.; Lee, S. S.; Balart, L.; Reindollar, R.; Reddy, R. K.; Wright, T. L.; Lin, A.; Hoffman, J.; De Pamphilis, J. *N. Engl. J. Med.* **2000**, *343*, 1673.
6. Dymock, B. *Emerg. Drugs* **2001**, *6*, 13.
7. Bartenschlager, R.; Lohmann, V. *J. Gen. Virol.* **2000**, *81*, 1631.
8. Bieroni, L.; Saltonini, E.; Fipaldini, C. *J. Virol.* **1997**, *71*, 4665.
9. Ingallinella, P.; Altamura, S.; Bianchi, E.; Talinani, M.; Ingenito, R.; Cortese, I.; De Francesco, R.; Steinkuhler, C.; Pessi, A. *Biochemistry* **1998**, *37*, 8906.
10. Kakiuchi, N.; Komoda, Y.; Komoda, K. *FEBS Lett.* **1998**, *421*, 217.
11. Chu, M.; Mierzwa, R.; Truumees, I. *Tetrahedron Lett.* **1996**, *37*, 7229.
12. Katz, B.; Clark, J.; Finer-Moore, J.; Jenkins, T.; Johnson, C.; Ross, M.; Luong, C.; Moore, W.; Stroud, R. *Nature* **1998**, *391*, 608.
13. *K_i' determinations*. HCV protease inhibition studies were performed in 50 mM HEPES buffer (pH 7.5), 50% glycerol, 0.005% polyoxyethylenesorbitan monolaurate (Tween-20), and 10% DMSO. A synthetic 17 amino acid NS4A surrogate (Ac-KKKGSVVIVGRIILSGR-$CONH_2$) was included in the assay at 40 μM. HCV protease (12 nM active sites, recombinant HCV protease consisted of the first 183 amino acids of the NS3 coding region) was incubated with inhibitor, present

at varying concentrations, for 30 min at room temperature (21–24 °C) in 96-well microtiter plates. Typically, the evaluation of a given inhibitor's potency was determined under two distinct sets of experimental conditions. In one set of assay conditions, $ZnCl_2$ was supplied at 12.5 μM. In parallel experiments, the inhibitor was evaluated without Zn(II) supplementation and with 25 μM EDTA included in the standard assay. After pre-incubation, reactions were initiated with the addition of the quenched fluorescence substrate, Ac-DED(Edans)EE-Abu[COO]-ASK(Dabcyl)-$CONH_2$ (final concentration was 1.5 μM) (Taliani, M.; Bianchi, E.; Narjes, F.; Fossatelli, M.; Urbani, A.; Steinkuhler, C.; De Francesco, R.; Pessi, A. *Anal. Biochem,* **1996**, *240*, 60). The hydrolysis of this substrate was monitored fluorometrically (filter pair: excitation 355 nm, emission 485 nm) using an FMAX Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The velocity of the HCV protease catalyzed reaction was obtained from the linear portion of the progress curves. Apparent inhibition constants, K_i', were calculated from the velocity data generated at the various inhibitor concentrations using the software package, Batch K_i (Biokin Ltd., Pullman, WA, USA) (Kuzmic, P.; Sideris, S.; Cregar, L. M.; Elrod, K. C.; Rice, K. D.; Janc, J. W. *Anal. Biochem.* **2000**, *281*, 62). Batch K_i provides a parametric method for the determination of inhibitor potency using a transformation of the tight binding inhibition model described by Morrison (Morrison, J. F. *Biochem. Biophys. Acta* **1969**, *185*, 269). A decapeptide was used as a positive control: H_2N-E-D-V-V-L-C-Tic-Nle-S-Y-OH: 298 nM in the presence of Zn^{2+}, 724 nm in the presence of EDTA, literature value: 340 nM, Landro, J. A.; Raybuck, S. A.; Luong, J. P. J.; O'Malley, E. T.; Harbeson, S. L.; Morgenstern, K. A.; Rao, G.; Livingston, D. J. *Biochemistry* **1997**, *36*, 9340.

14. Yeung, K.-S.; Meanwell, N.; Qiu, Z.; Hernandez, D.; Zhang, S.; McPhee, F.; Weinheimer, S.; Clark, J.; Janc, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2355.
15. Katz, B.; Luong, C. *J. Mol. Biol.* **1999**, *292*, 669.
16. Barbato, G.; Cicero, D.; Nardi, M.; Steinkuhler, C.; Cortese, R.; Francesco, R. D. *J. Mol. Biol.* **1999**, *289*, 371.
17. Cicero, D.; Barbato, G.; Koch, U.; Ingallinella, P.; Bianchi, E.; Nardi, M.; Steinkuhler, C.; Cortese, R.; Matassa, V.; Francesco, R. D.; Pessi, A.; Bazzo, R. *J. Mol. Biol.* **1999**, *289*, 385.
18. LaPlante, S.; Cameront, D.; Aubry, N.; Lefebre, S.; Kukolj, G.; Maurice, R.; Thibeault, D.; Lamarre, D.; Llinas-Brunet, M. *J. Biol. Chem.* **1999**, *274*, 18618.