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Inhibition of Human Cytomegalovirus Protease by Monocyclic β-Lactam Derivatives: Kinetic Characterization Using a Fluorescent Probe

Pierre R. Bonneau,*,† Firoz Hasani,† Céline Plouffe,† Eric Malenfant,† Steve R. LaPlante,† Ingrid Guse,† William W. Ogilvie,† Raymond Plante,† Walter C. Davidson,‡ Jerry L. Hopkins,‡ Maurice M. Morelock,‡ Michael G. Cordingley,† and Robert Déziel†

Contribution from the Departments of Biological Sciences and Chemistry, Boehringer Ingelheim (Canada) Ltd., Bio-Méga Research Division, Laval, Québec H7S 2G5, Canada, and Departments of Analytical Chemistry and Biology, Boehringer Ingelheim Pharmaceuticals Inc., Research and Development Center, 175 Briar Bridge Road, Ridgefield, Connecticut 06877

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Abstract: Recent reports have demonstrated the potential of monocyclic β-lactam derivatives as inhibitors of human cytomegalovirus (HCMV) protease. Investigation of the mechanism of inhibition by NMR and mass spectrometry has revealed the presence of an acylenzyme intermediate suggesting that β-lactams are hydrolyzed by the enzyme and cause inhibition by competing with substrate. The potential of a fluorogenic β-lactam derivative for convenient kinetic characterization of this mechanism has been evaluated using 4*S*-(4-methylumbelliferone)-3*R*-methylazetidin-2-one-1-carboxylic acid (4-methylpyridyl) amide (**1**). Upon acylation of the enzyme, the fluorescent umbelliferone moiety is released, allowing for continuous monitoring of the hydrolytic process. Examination of a series of progress curves by numerical analysis has provided valuable information on acylation and deacylation rates which relate to the IC_{50} values observed for β-lactams. More importantly the potential of compound **1** as an active site titrating agent for HCMV protease has been exploited, and a simple protocol for rapid determination of active enzyme is described. The data are consistent with the HCMV protease dimer being composed of two functional active sites. This titrating agent represents an important tool that should significantly facilitate the characterization of this novel enzyme.

Introduction

Human cytomegalovirus (HCMV, a β-herpesvirus) is a serious pathogen in immunocompromised individuals, including AIDS patients, neonates, and organ transplant recipients.[1,2] As a member of the herpesviridae family, HCMV encodes a serine protease which is involved in capsid assembly and is essential for the production of infectious virions.[3−5] X-ray structure determination of the enzyme has revealed a novel fold and an unusual catalytic triad which appears unique to herpesvirus proteases.[6−9] HCMV protease therefore represents both an attractive target for the development of new antiviral agents and an excellent candidate for further studies on the catalytic machinery of these serine proteases.

We have shown recently,[10−12] along with other laboratories,[13] the potential of monocyclic β-lactams as inhibitors of HCMV protease. Similar compounds have been studied in depth as potent inhibitors of mammalian serine proteases such as human leukocyte elastase (HLE) and thrombin.[14−18] These studies have demonstrated that β-lactams can be processed by these enzymes

* To whom correspondence should be addressed. Phone: (450) 682-4640. Fax: (450) 682-8434.

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‡ Boehringer Ingelheim Pharmaceuticals Inc.

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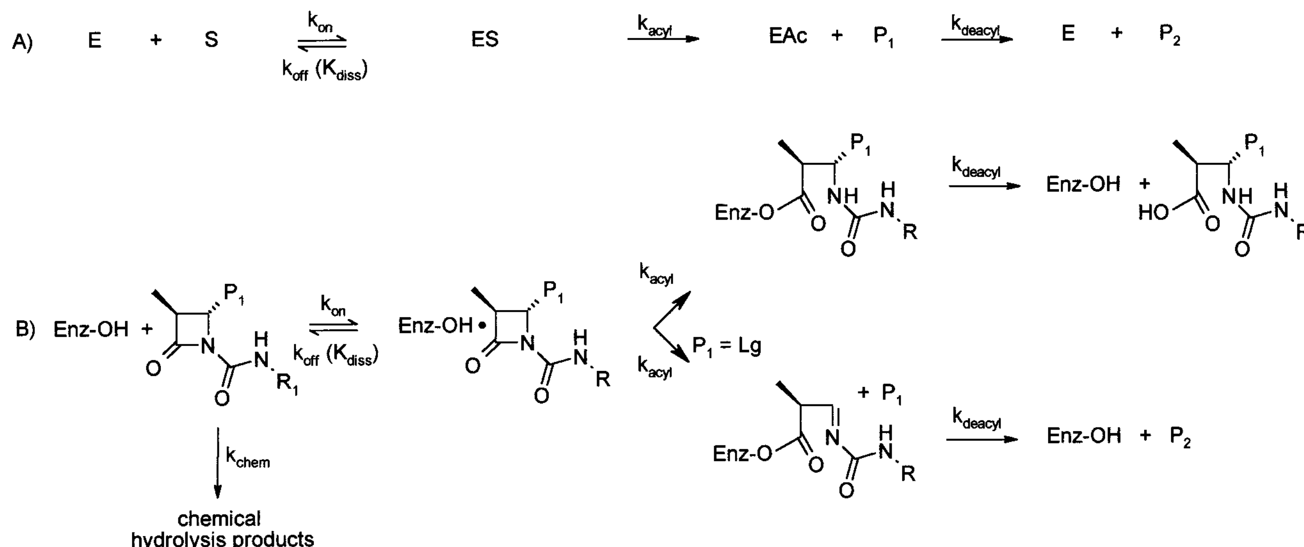

Figure 1. (A) General mechanism of serine protease-catalyzed hydrolysis. (B) Model for HCMV protease-catalyzed hydrolysis of β-lactam derivatives.

through a mechanism related to the familiar substrate hydrolysis pathway archetypal to serine proteases (Figure 1A,B). In this scheme, free enzyme (E) and substrate (S) are in equilibrium with a noncovalent Michaelis complex (ES). Acylation of the ES complex then leads to the formation of the acylenzyme intermediate (EAc). Extensive mechanistic studies with HLE and several monocyclic β-lactams have revealed the central role played by the acylenzyme during inhibition.[14,16,19,20] Although several pathways have been proposed from this intermediate depending on the exact nature of the β-lactam, the overall inhibition is generally believed to originate from the inability of HLE to undergo efficient deacylation once bound to these compounds. Indeed very slow recovery of enzymatic activity has been observed with a half-life up to 15 h, rendering the apparent inhibition as essentially irreversible in extreme cases.[19]

By extension from these previous studies, the inhibition of HCMV protease by β-lactams is likely to proceed according to the same general mechanism. This report presents physical evidence supporting the presence of an acylenzyme intermediate during β-lactam inhibition of HCMV protease. The characterization of the fluorescent β-lactam 4*S*-(4-methylumbelliferone)-3*R*-methylazetidin-2-one-1-carboxylic acid (4-methylpyridyl) amide (**1**) as a tool for the detailed analysis of the overall mechanism of inhibition is also described. We show that the kinetic characterization of compound **1** not only lends additional support to the proposed mechanism of inhibition but also provides the first access to an active site titration protocol of HCMV protease and adds to the numerous assay methodologies[21−28] currently available to study this important enzyme.

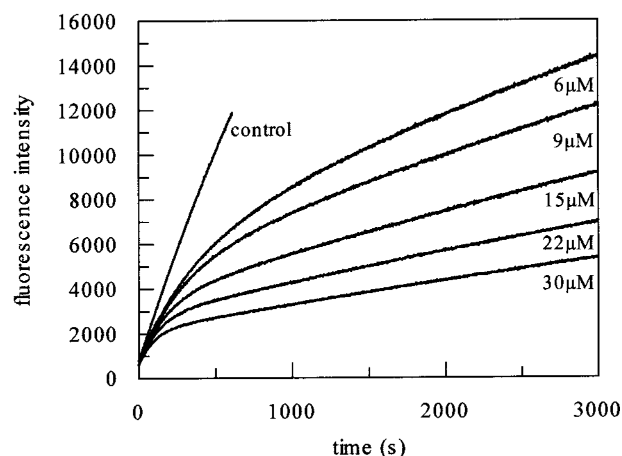

Figure 2. Time-dependent inhibition of HCMV protease by β-lactam **2** (6−30 μM). Progress curves in the presence of inhibitor were monitored up to 30% conversion of 5 μM of the substrate *N*-Acetyl-Tbg-Tbg-Asn(NMe)$_2$-Ala-AMC.

Results

The effect of a typical monocyclic β-lactam derivative (compound **2**) on the hydrolysis of the specific substrate *N*-Acetyl-Tbg-Tbg-Asn(NMe)$_2$-Ala-AMC is shown in Figure 2. The inhibition of HCMV protease is time-dependent, the progress curves revealing a slow onset of inhibition followed by a steady-state rate. The inability of this compound to completely abolish activity with time is indicative of the reversible nature of the inhibition. Indeed saturation of the

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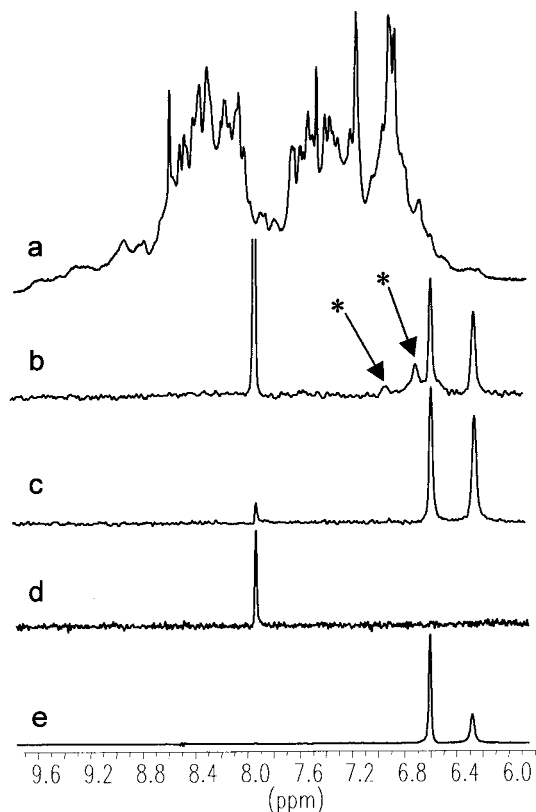
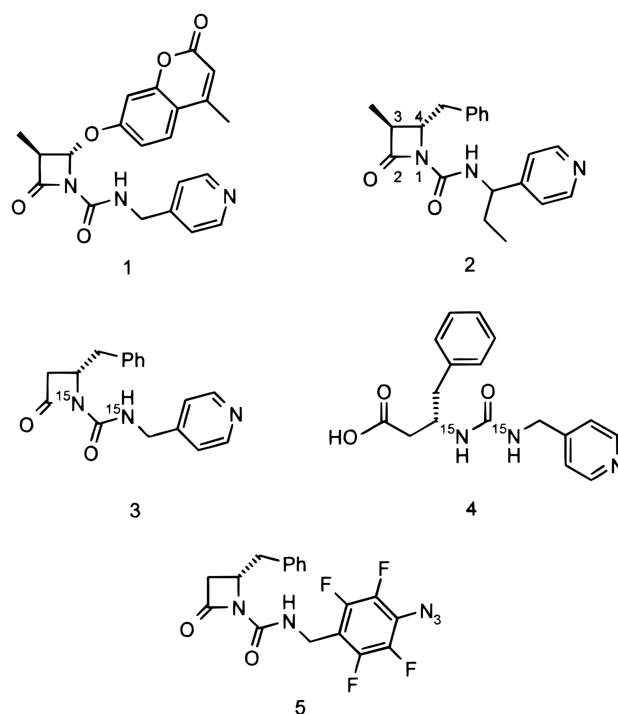


Figure 3. Downfield subregion of ^{1}H NMR spectra; (a) normal ^{1}H spectrum taken within minutes after mixing compound **3** with HCMV protease at a 3:1 ratio; (b) ^{15}N-filtered ^{1}H spectrum of the same sample after 1 h incubation. (c) ^{15}N-filtered ^{1}H spectrum of the same sample after 12 h incubation; (d) ^{15}N-filtered ^{1}H spectrum of free **3** (no protease); (e) ^{15}N-filtered ^{1}H spectrum of **4** (base-hydrolyzed form of free **3**).

enzyme followed by column dialysis of the excess inhibitor resulted in a progressive recovery of activity within minutes (data not shown). Moreover the general features of the inhibition curves remained unchanged with β-lactams displaying a leaving group substituent at C-4. This suggested that the overall inhibition did not depend on the departure of product P_1 and formation of a reactive imine moiety.

Despite its unique fold and unusual His-Ser-His catalytic triad, HCMV protease is expected to operate in a manner similar to other members of the serine protease family. Support for this has been provided by the recent X-ray structure of the enzyme complexed with a mechanism-based peptide inhibitor which shows the anticipated tetrahedral intermediate stabilized by an oxyanion hole.[29] The general model for substrate hydrolysis by this class of enzyme would then appear attractive to explain β-lactam inhibition of HCMV protease (Figure 1A,B). In this scenario, the β-lactam and the enzyme are involved in an equilibrium ($K_{\mathrm{diss}} = k_{\mathrm{off}}/k_{\mathrm{on}}$) with a noncovalent Michaelis complex. This complex proceeds toward acylenzyme formation upon attack of the activated serine residue of the enzyme on the ring carbonyl of the β-lactam at a rate k_{acyl} (with concomitant release of product P_1 representing the C-4 leaving group if present). Upon deacylation of the P_2 moiety, the enzyme is reactivated at a rate k_{deacyl}. The fact that none of the β-lactams with a C-4 leaving group were capable of causing irreversible inhibition argues against covalent bond formation between the histidine residue of the active site and the imine intermediate formed upon elimination of P_1, a pathway otherwise known as "double-hit mechanism".[18,30] Ring opening of the β-lactam by chemical hydrolysis at an observed rate k_{hydr} is also shown in Figure 1B as an alternate path by which the leaving group P_1 at C-4 can be produced spontaneously. The apparent inhibition may thus be understood in terms of a competition for enzyme hydrolysis between a β-lactam and a specific substrate. Support for this mechanism is presented below and includes NMR and mass spectrometry data which convincingly demonstrate the formation of an acylenzyme intermediate.

Physical Evidence of an Acylenzyme Intermediate. NMR spectroscopy was used to investigate the structure of β-lactams when bound to HCMV protease. Compound **3** was synthesized such that the two nitrogens of the urea moiety were ^{15}N labeled. ^{1}H and ^{15}N-filtered ^{1}H NMR spectra of **3** in the presence of HCMV protease were acquired with a molar excess of **3** over the enzyme. Figure 3a shows the predominance of protease resonances in a normal ^{1}H spectrum. Application of the ^{15}N-filtered ^{1}H NMR technique reveals only the resonances of β-lactam hydrogens which are covalently attached to an ^{15}N atom (Figure 3b). The resonance at 7.9 ppm corresponds to free **3**, while the two relatively sharp resonances at 6.3 and 6.6 ppm belong to compound **4**, the product of processing of **3** by the enzyme. These chemical shifts agree with control spectra of free **3** and free **4** shown in Figure 3d,e. Evidence of the protease-bound form is observed in Figure 3b as two relatively broad resonances at 6.75 and 6.95 ppm (labeled with stars). Two resonances are expected for **3** in a predominant acylenzyme complex since both ^{15}N nitrogens should be protonated (ring-opened form). These transient resonances are no longer observed after 12 h, where **3** has been almost completely converted by protease (Figure 3c). These data collectively support the hypothesis that β-lactams are processed by HCMV protease through the formation of an acylenzyme intermediate.



The existence of this intermediate was also investigated by mass spectrometry. Reasoning that acylation may improve

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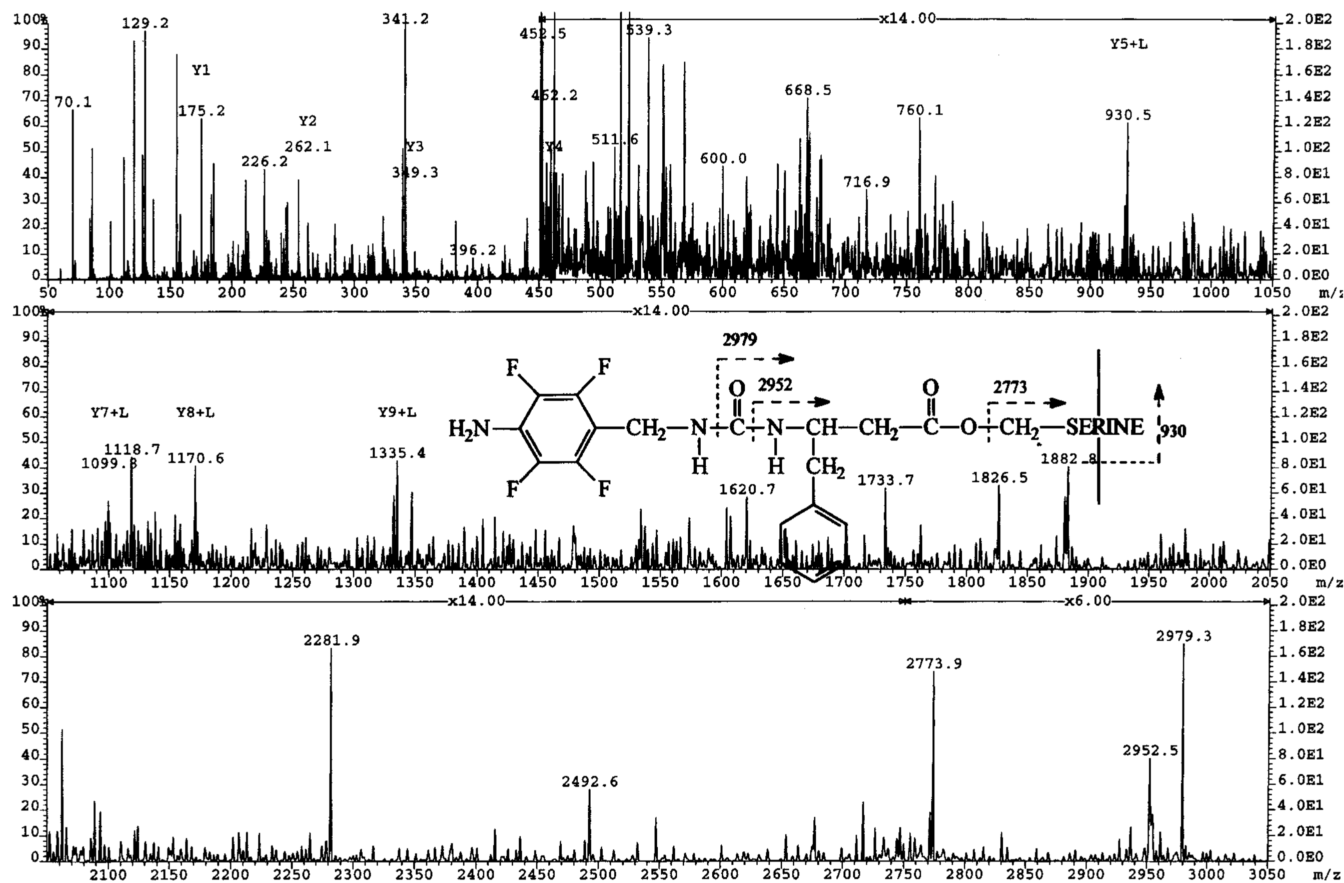

Figure 4. MALDI high-energy MS/MS spectrum of tryptic digest ($MH^+ = 3172$).

chances for successful affinity labeling of the enzyme, the azido β-lactam derivative **5** was designed to help identify the orientation of the side chains. Following UV irradiation, spectrometric analysis of the tryptic fragments of the complex may reveal either one or both of two possible covalent bonds (i.e., acylation and nitrene insertion). Compared with the control, the labeled sample showed additional LC/MS responses at m/z = 1586 and 1057, consistent with the doubly and triply charged molecular ions of labeled residues 110−136. The peak maximum for the triply charged ion corresponded to a molecular weight of 3172.2, which is consistent with the addition of the label via acylation of a serine residue accompanied by reaction of the nitrene with water rather than with the protein. MALDI MS/MS analysis of the HPLC peak corresponding to the mass of 3172 displayed several daughter ions (2979.3, 2952.5, 2773.9) resulting from fragmentation of the ring-opened form of **5** as depicted in Figure 4. The ion at 2773.9 was assigned to cleavage of a serine oxygen, thus further supporting the acylation mechanism. Moreover, analysis of the Y series of fragmented peptides in the region Tyr128-Arg136 showed a sudden increase in mass at residue Ser132 corresponding to that of the added label (Y_1, Arg136; Y_2, Ser135-Arg136, ...; see Figure 4). These results are consistent with previous observations[13] and convincingly show that acylation occurred at the active site Ser132. The absence of nitrene insertion to the protein suggests that the flexibility of the inhibitor side chains within the acylenzyme may be sufficient to prevent labeling. So far this interpretation has been consistent with our inability to resolve the electronic density of the ligand in X-ray experiments involving HCMV protease complexed with various β-lactam derivatives.[31]

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Kinetic Analysis of Hydrolysis of Compound 1. After establishing that HCMV protease processes monocyclic β-lactams through an acylenzyme intermediate according to Figure 1B, the kinetic analysis for the enzymatic hydrolysis of β-lactam **1** was undertaken. This derivative only differs from other β-lactams such as **2** in that it allows for continuous monitoring of the hydrolytic process by the release of the fluorescent umbelliferone leaving group at C-4 upon ring opening. Thus, no other substrate is required to follow the effect of the β-lactam on the enzyme, making compound **1** a convenient probe to kinetically characterize the general mechanism of action of β-lactam inhibitors.

β-Lactam **1** belongs to a class of activated β-lactams that chemically hydrolyze rapidly due to the combined effects of the urea moiety and the C-4 leaving group. These groups substantially increase the electrophilicity of the ring carbonyl and render the β-lactam more susceptible to ring opening with concomitant release of the C-4 substituent. The rate of chemical hydrolysis of **1**, k_{hydr}, as measured from the appearance of the fluorescent umbelliferone leaving group in absence of HCMV protease was 9.7×10^{-5} s^{-1} in the assay conditions (Figure 5). The experiment was repeated in the presence of diisopropylfluorophosphate(DFP)-inactivated HCMV protease and resulted in a similar k_{hydr} value, indicating that no general protein catalysis was at play. To prevent significant inner-filter effects from the increasing concentration of 4-methylumbelliferone during the reaction, an excitation wavelength of 395 nm (away from λ_{max} of 357 nm) was selected. Excitation at 395 nm with emission at 446 nm produced linear fluorescence intensities up to 60 μM 4-methylumbelliferone (data not shown). The umbelliferone moiety was also found to be photostable for over 1 h upon excitation at 395 nm, thus permitting long exposure.

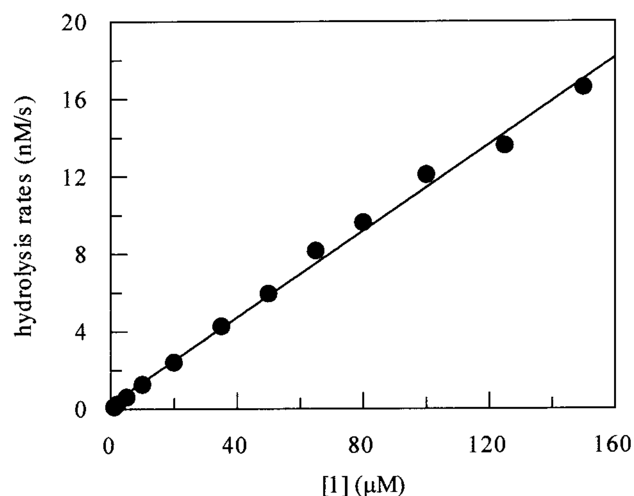

Figure 5. Chemical hydrolysis of β-lactam **1** in the absence of HCMV protease. Initial rates of formation of 4-methylumbelliferone as monitored by fluorescence increase upon excitation at 395 nm are plotted against the concentration of **1**. Buffer conditions were 50 mM Tris/HCl, pH 8, 0.5 M Na_2SO_4, 50 mM NaCl, 0.1 mM EDTA, 1 mM TCEP, and 3% v/v DMSO.

The progress curves of hydrolysis of compound **1** in the presence of 3 and 6 μM HCMV protease are depicted in Figure 6A,B (solid lines). The enzyme concentrations used were estimated on the basis of the UV determination made by the method of Scopes[32,33] and therefore represent total HCMV protease concentration. The enzyme concentrations in this study were chosen to obtain rates of enzymatic hydrolysis significantly above those of chemical hydrolysis. A broad range of concentrations of **1** was used from 1 to 200 μM. At the lowest concentrations, hydrolysis proceeded to near completion over 45 min in an apparent first-order process, while at the higher concentrations (low substrate conversions) the biphasic nature of the curves became apparent. It is interesting to note the resemblance between these progress curves in which the β-lactam **1** is the sole substrate and those obtained when the β-lactam **2** is acting as an inhibitor in the presence of the substrate *N*-Acetyl-Tbg-Tbg-Asn(NMe)$_2$-Ala-AMC (Figure 2). This is consistent with β-lactams acting as competitive substrates. A stopped-flow approach using a fast-kinetic apparatus was also used to visualize the initial moments of the hydrolytic process. Figure 7 reveals that initial hydrolysis proceeded linearly with no sign of upward curvature. Such linearity is consistent with a rapid initial binding event between the enzyme and **1** (large k_{on} and k_{off}), making the instantaneous equilibrium hypothesis a valid assumption for further characterization of the mechanism.

Discussion

Two factors precluded the use of classical kinetic treatment of these progress curves to extract the relevant kinetic parameters. First the enzyme concentrations used were in a range comparable to that of **1**, i.e., $[S]_{free} \neq [S]_0$. Second, the rate of chemical hydrolysis could not be subtracted easily from the progress curves and, albeit small, became sufficiently important toward the end of the reaction to require appropriate corrections. For these reasons, the data from each set of progress curves ($[E]_0 = 3$ and 6 μM) were analyzed by a numeric computational method applied to a system of differential rate equations as defined by the kinetic model of Figure 1B (for more details see Supporting Information). Such an approach has already been used successfully for the kinetic characterization of inhibitors of the HIV protease homodimer.[34] The equations were derived by making the following assumptions: (1) instantaneous equi-

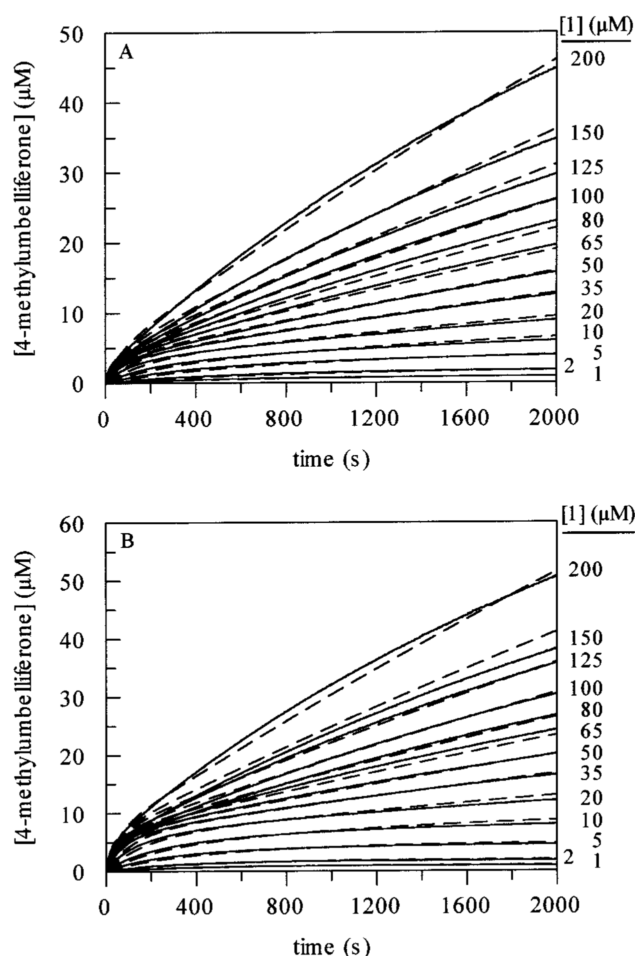

Figure 6. Progress curves for HCMV protease-catalyzed hydrolysis of 1−200 μM compound **1** using (A) 3 μM and (B) 6 μM total enzyme concentration (determined by the UV205/280 method):[32,33] (—) experimental curve, (- - -) predicted curve (see Experimental Section for details).

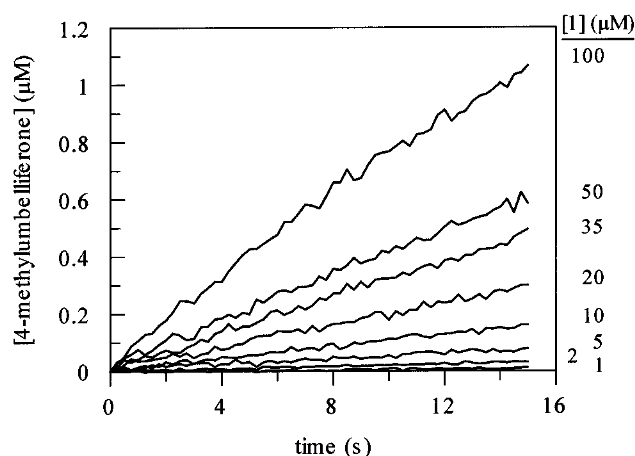

Figure 7. Progress curves under stopped-flow conditions for HCMV protease-catalyzed hydrolysis of 1−100 μM compound **1** using 3 μM total enzyme concentration (determined by the UV205/280 method).[32,33]

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Table 1. Fitted Kinetic Parameters and Active Site Concentrations for Both Sets of Progress Curves as a Function of Total HCMV Protease[a]

parameter	$[E]_0 = 3\ \mu M^b$	$[E]_0 = 6\ \mu M^b$
$[\text{active site}]_0$	$4.04 \pm 0.01\ \mu M$	$6.86 \pm 0.02\ \mu M$
K_{diss}	$66 \pm 2\ \mu M$	$62 \pm 2\ \mu M$
k_{acyl}	$(2.17 \pm 0.06) \times 10^{-2}\ s^{-1}$	$(2.42 \pm 0.06) \times 10^{-2}\ s^{-1}$
k_{deacyl}	$(4.48 \pm 0.05) \times 10^{-4}\ s^{-1}$	$(4.14 \pm 0.04) \times 10^{-4}\ s^{-1}$
k_{hydr}	$(1.114 \pm 0.001) \times 10^{-4}\ s^{-1}$	$(1.161 \pm 0.001) \times 10^{-4}\ s^{-1}$

[a] Values obtained by numerical analysis of the progress curves of Figure 6A,B and according to the equations defining the model of Figure 1B (see Experimental Section for details). Standard errors are shown. [b] Total monomer concentration based on UV 205/280 ratio.[32,33]

librium between E and S, (2) irreversible acylation, and (3) relatively fast release of P_1 concomitant with acylation. These assumptions were justified by the lack of upward curvatures during stopped-flow experiments, the thermodynamically unfavorable recyclization of the β-lactam ring,[35] and the relatively low contribution to binding of the substituent at position C-4, making its release an unlikely rate-participating step.[10−12] Finally it has been shown that dimerization of HCMV protease is essential for catalytic activity.[36,37] However it is not yet clear whether the dimer contains one or two functional active sites. Although the present study may provide further insights into this question, our current model makes no assumption as to the functionality of each monomer within the dimer or cooperativity between them. Therefore the symbol E in Figure 1A should represent one active site rather than being interpreted as an enzyme dimer. Since the high concentrations of both protease and salt used in this study ensure that the enzyme predominantly exists in the dimeric form,[38] it is more appropriate to refer to the total concentration of active sites rather than concentration of active enzyme.

Significance of the Rate Constants to the Mechanism Of Action of β-Lactams. Values for K_{diss}, k_{acyl}, k_{deacy}, k_{hydr}, and $[\text{active site}]_0$ (Table 1) along with the predicted traces superimposed over the experimental data were obtained for both sets of progress curves (Figure 6A,B). The fitted curves (dotted lines) are generally in good agreement with the experimental ones (solid lines), a fact reflected by the similarity of the kinetic parameters generated from both sets of data. The predicted rate of chemical hydrolysis, k_{hydr}, corresponds to the rate previously determined in the absence of HCMV protease ($1.1 \times 10^{-4}\ s^{-1}$ compared to $9.7 \times 10^{-5}\ s^{-1}$). More importantly, the predicted active site concentrations are close to those determined by the photometric method for the total monomer concentrations. This not only implies that the enzyme stock is predominantly in the active form but is consistent with the HCMV protease dimer being composed of two functional active sites. This is also in agreement with the observation that in the X-ray structure of the inhibited enzyme both monomeric units are occupied by a mechanism-based inhibitor.[29] However, more information is required to determine whether both sites are completely independent from each other or if some degree of cooperativity exists. It has already been suggested that substrate binding enhances dimerization,[38] and we have demonstrated recently that the protease undergoes a change in conformation upon binding of peptidic inhibitors.[39] Although no conformational change has been detected for β-lactams,[40] one cannot yet rule out the possibility that binding of such ligands to one monomer could modulate both the affinity and the activity of the adjacent site through a tightening of the complex. This kind of mechanism may be relevant in the context of viral capsid maturation and represents a potential new strategy for drug design.

The rates of acylation (k_{acyl}) and deacylation (k_{deacyl}) were determined to be 0.023 s^{-1} and 0.000 43 s^{-1}, respectively. At steady state, the ratio [ES]/[EAc] given by k_{deacyl}/k_{acyl} is 0.02, indicating that the acylenzyme intermediate is predominant during the linear portion of the progress curves and constitutes approximately 98% of the bound enzyme. The dissociation constant, K_{diss}, between E and **1** was estimated to be 64 μM. When assessed as an inhibitor using a previously described procedure,[41] compound **1** displayed an IC_{50} value of 0.6 μM. Considering that both IC_{50} and K_{diss} represent the concentration of compound at which half of the total enzyme is bound, the discrepancy between these values may appear inconsistent. However this resolves itself when it is considered that the IC_{50} values of β-lactams were obtained at steady state and must therefore reflect the global K_M of the β-lactam probe rather than K_{diss}. This global K_M is obtained by the application of the mass balance equation on E_o and the steady-state approximation to both ES and EAc;

$$K_M = [(k_{acyl} + k_{off})/(k_{on})]/[(k_{deacyl})/(k_{acyl} + k_{deacyl})] \quad (1)$$

In this particular case, the equation simplifies to $K_{diss}(k_{deacyl}/k_{acyl})$ since $k_{off} > k_{acyl} > k_{deacyl}$. Therefore, the calculated IC_{50} for **1** obtained from the values listed in Table 1 is 1.2 μM, which compares well with the experimental value of 0.6 μM.

Since the mechanism of action described above probably applies to other β-lactam derivatives, the acylation and deacylation steps can be considered fundamental to the hydrolytic process, whether the C-4 position is occupied by a leaving group or not. It is interesting to relate the variation of these individual kinetic parameters with the IC_{50} values observed over a broad range of β-lactams. In the absence of a leaving group at C-4, the electrophilicity of the ring carbonyl is reduced, making it more stable to hydrolysis but also less susceptible to nucleophilic attack (i.e., acylation) by the catalytic serine residue. Indeed, extensive work on a variety of β-lactam derivatives has shown that such a decrease in the electrophilic character of the carbonyl group can lead to large reductions in chemical hydrolysis and methanolysis rates.[42−44] Of particular interest is the influence of the C-4 substituent, which can reduce the hydrolysis rates up to 100-fold as its leaving group character is attenuated.[44] If such a reduction in acylation rate is operative in the case of HCMV protease-catalyzed hydrolysis of β-lactam derivatives without an activating leaving group at C-4, the gap between

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k_{acyl} and k_{deacyl} would diminish accordingly, leading to increased concentrations of the ES complex during steady state relative to EAc. Assuming only a 10-fold reduction of k_{acyl}, from 0.023 to 0.0023 s^{-1}, for such β-lactams (given similar K_{diss} and k_{deacyl} as for **1**), the proportion of noncovalent ES complex at steady state may reach up to 20% of the total bound enzyme by virtue of the increasing ratio k_{deacyl}/k_{acyl} = [ES]/[EAc] (i.e., 0.00043 s^{-1}/0.0023 $s^{-1} \approx 0.2$). Accordingly, the potency should in turn decrease 10-fold given the equation $IC_{50} \approx K_{diss}(k_{deacyl}/k_{acyl})$. This trend was indeed observed for pairs of β-lactam derivatives in which the leaving group was transformed into a nonleaving moiety by merely substituting the C-4 heteroatom with carbon. It may therefore be concluded that a large part of the inhibitory potency of β-lactams is attributed to the magnitude of k_{deacyl}/k_{acyl} (such that IC_{50} values also decrease with this ratio). On the basis of this simple relationship, modulation of k_{deacyl} toward smaller values would also contribute to higher potency. This was observed for β-lactam inhibition of HLE where reactivation of enzyme occurred with observed rate constants ranging from 1×10^{-5} to 1×10^{-4} s^{-1}, rendering the inhibition essentially irreversible in extreme cases.[19] For HCMV protease, the value of 4.3×10^{-4} s^{-1} for k_{deacyl} still permits reversible inhibition to be observed but is only 2−3 orders of magnitude greater than the expected rate of base-catalyzed hydrolysis of a normal aliphatic ester at pH 8[45,46] in the presence of a large amount of Na_2SO_4.[47] This suggests that the enzyme does not participate heavily to its own reactivation[40] and that the acylated form of β-lactam **1** is not involved in strong interactions that can contribute to the slowing down of its release. The unsuccessful photoaffinity labeling experiment with compound **5** and the difficult crystallographic resolution of protease-complexed β-lactams[31] are in agreement with a loosely bound intermediate. As a result of these weak interactions, it is likely that most β-lactams with backbone structures related to compound **1** deacylate with similar rates. Given the maximal value of k_{acyl} (which has likely been reached with highly activated β-lactams such as **1**) and the limiting value of k_{deacyl}, modulating K_{diss} appears to be the only option available to enhance the potency of β-lactam derivatives. Thus, β-lactam **1** with a dissociation constant of 64 μM from HCMV protease clearly represents a good starting point for the design of more potent, scaffold-like molecules that may exploit new binding opportunities.

Conclusion

We have demonstrated in this study that monocyclic β-lactam derivatives reversibly inhibit HCMV protease by acting as competing substrates through the formation of an acylenzyme intermediate. While ^{15}N-filtered ^{1}H NMR experiments strongly suggested the existence of such an intermediate, mass spectrometry unambiguously revealed acylation of the enzyme at the expected activated serine 132 residue. Additional support for the proposed mechanism was provided by the fluorogenic β-lactam probe 4*S*-(4-methylumbelliferone)-3*R*-methylazetidin-2-one-1-carboxylic acid (4-methylpyridyl) amide **(1)**, whose kinetic characterization by numerical analysis was found to be consistent with the general model of serine protease hydrolysis. The time-dependency of inhibition was related to a relatively slow acylation rate followed by an even slower, rate-determining, deacylation step. Overall, the data was found to be consistent with the HCMV protease dimer being composed of two functional active sites. Studies are currently under way to investigate possible cooperativity between these sites. This fluorescent probe not only represents an important tool that should significantly facilitate the characterization of the intrinsic properties of this novel protease but also allows for the development of improved scaffold-like inhibitors of this enzyme based on the exploitation of a noncovalent, micromolar affinity.

Experimental Section

Materials for the synthesis were from commercial sources and used without further purification. 4-Methylumbelliferone was purchased from Sigma. Compound **3** was synthesized from l-phenylalanine ($^{13}C_9$;^{15}N) (Cambridge Isotope Laboratory, Andover, MA) and from phthalimide-^{15}N, potassium derivative (Sigma-Aldrich, Mississauga, ON, Canada). 3*R*-4-Acetoxyazetidin-2-one,[30] pyridin-4-ylmethylcarbamic acid phenyl ester,[10] 4*S*-benzylazetidin-2-one,[10] 4-azido-2,3,5,6-tetrafluorobenzylamine,[48,49] and the fluorogenic substrate *N*-Acetyl-Tbg-Tbg-Asn-$(NMe)_2$-Ala-AMC[27] (Tbg, *tert*-butylglycine; AMC, 7-amino-4-methylcoumarin) were synthesized as previously described. All studies were carried out using the HCMV protease mutant Ala143Gln, which eliminated the problem of autoproteolysis.[22] The enzyme was expressed and purified as described earlier[39] and concentrated in storing buffer (NaOAc 20 mM pH 5, DTT 1 mM, EDTA 0.1 mM). The concentration of protein was determined by spectrophotometry using a Cary 1E UV−vis photometer (Varian, Victoria, Australia).

Fluorescence measurements were performed in quartz cuvettes on a SLM-Aminco model 8100 spectrophotometer (Spectronics Instrument, Rochester, NY). Stopped-flow experiments were performed using the SFA-20 Rapid Kinetic Accessory from Hi-Tech Scientific (Salisbury, U.K.). Illumination studies were performed with a portable 254/365 nm UV lamp FBUVL580 (Fisher Scientific).

One-dimensional ^{1}H NMR spectra were acquired at 25 °C on a Bruker DRX 800 MHz spectrometer (Milton, ON, Canada). Data were recorded with 11 185 Hz sweep width, 2K time domain points, relaxation delay of 1.3 s, and averaging of 2K transients. Suppression of the solvent signal was achieved by the use of the WATERGATE method.[50] One-dimensional ^{15}N-filtered ^{1}H HSQC spectra[51] were recorded with similar parameters. Resonances of hydrogens attached to ^{15}N-labeled nitrogens were filtered using a delay that selected a 90 Hz $^{1}H-^{15}N$ coupling. ^{15}N-decoupling was applied during data acquisition. Data were processed using Bruker WinNMR software. A phase-shifted sinebell window function was applied to data sets followed by zero-filling and Fourier transformation.

LC/MS experiments were conducted on an API III instrument (SCIEX, Thornhill, Canada) operated in electrospray mode. HPLC was performed with a Vydac (Hesperia, CA) 300 Å, C18 15 cm × 0.1 cm column operated at 40 μL/min flow rate (Mobile phase A: 95% v/v H_2O, 5% acetonitrile, 0.06% TFA. Mobile phase B: 5% H_2O, 95% acetonitrile, 0.052% TFA). The HPLC gradient was held at 0% B for 3 min and then increased to 40% B at 50 min and to 100% B at 65 min until 75 min with detection at 215 nm. Mass monitoring was performed with a 2 amu window, 0.1 amu step size, and a dwell of 50 ms. MALDI MS/MS experiments were performed on an Autospec OATOF instrument (Micromass, Manchester, U.K.) employing high energy collision conditions. The HPLC procedure described above was used to isolate the component having MW = 3172. The isolate was placed on the MALDI probe along with 2,5-dihydroxybenzoic acid in 10% ethanol matrix. Data were acquired for 14 min in continuum mode and averaged into a single spectrum.

Kinetic data analysis was performed by applying ordinary nonlinear least-squares regression techniques using the Marquardt minimization method. The parameters estimated were [active site]$_0$, K_{diss}, k_{acyl}, k_{deacy}, and k_{hydr} using the model described in Figure 1B. All of the data were

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analyzed using the SAS statistical software system (version 6.12 for Windows, SAS Institute Inc., Cary, NC) on a Dell Optiflex GXa computer (see Supporting Information).

Determination of Protein Concentration. Protein concentration was based on the method of Scopes[32,33] using the ratio of the absorbance at 205 and 280 nm. Briefly, two series of HCMV protease dilutions were prepared in 5 mM phosphate buffer, pH 7, containing 50 mM Na_2SO_4. Absorbance of the concentrated series was determined at 280 nm, while that of the diluted series was determined at 205 nm. Each series of absorbance was plotted against the volume of stock enzyme used, and the slopes of these linear plots were determined. Special attention was paid to the preparation of appropriate blanks for zeroing the instrument. The extinction coefficient was calculated using the following equation:

$$E_{205}^{1\ \mathrm{mg/mL}} = 27.0 + 120(\text{slope at 280 nm/slope at 205 nm}) = 31.9 \quad (2)$$

This represents the extinction coefficient of a 1 mg/mL solution of HCMV protease at 205 nm after correction for the tyrosine and tryptophan content. Using this procedure, the total concentration of the preparation of HCMV protease Ala143Gln used in this study was estimated at 5.22 mg/mL (186 μM monomer concentration assuming a molecular mass of 28 099 Da). Unless noted otherwise, the concentration of HCMV protease is expressed in terms of total monomer concentration and is based on the above UV determination. Protein determination by the method of Bradford[52] gave concentrations of HCMV protease repeatedly 2- to 3-fold higher than those obtained by the more accurate and reliable method of Scopes.[53]

NMR Sample Preparation. Three samples were prepared for NMR studies. The buffer used for all samples was 50 mM Tris/HCl-d_{11}, pH 7, 0.5 M Na_2SO_4, 50 mM NaCl, 1 mM EDTA-d_{16}, 0.5 mM tris(2-carboxylethyl)phosphine hydrochloride (TCEP) containing 10% v/v D_2O. The first sample was prepared by adding compound **3** (from a DMSO-d_6 stock) and HCMV protease to the buffer at final concentrations of 2 and 0.65 mM, respectively (**3**/protease ratio of 3:1). The second sample was prepared by adding compound **3** to the buffer at a final concentration of 650 μM (no enzyme). The last sample was prepared by adding compound **4** (base-hydrolyzed form of compound **3**) to the buffer at a final concentration of 1.9 mM. The hydrolyzed form was made by adding sodium hydroxide to a solution containing compound **3**, which resulted in complete opening of the β-lactam ring.

UV Activation of Compound 5 and Tryptic Digest. Concentrated HCMV protease was dialyzed against 50 mM Tris/HCl, pH 8, 0.5 M Na_2SO_4 , 50 mM NaCl, and 0.1 mM EDTA. To 131 μL of dialysis buffer in a light-protected tube was added 57 μL of dialyzed enzyme, 6 μL of DMSO, and 6 μL of a 10 mM solution of compound **5** in DMSO. The mixture was incubated for 1 h at room temperature. The sample was then transferred in the dark into a quartz cuvette with the largest surface area positioned 5 cm away from a portable UV lamp. The sample was illuminated at 254 nm for 3 min in the dark and immediately frozen at −80 °C until digestion with trypsin. A control experiment in the absence of **5** was also performed and treated as above. After lyophilization the samples were dissolved in 25 μL of 8 M urea containing 0.4 M ammonium bicarbonate. The samples were then reduced by adding 5 μL of 45 mM dithiothreitol (DTT) and incubating at 50 °C for 15 min. After cooling to room temperature, 5 μL of 100 mM iodoacetamide was added, and the samples were further incubated at 50 °C for 15 min. After cooling to room temperature, 61.7 μL of water was added along with 3.3 μL of a 1 mg/mL solution of trypsin (TPCK-treated from Worthington) prepared in 1 mM hydrochloric acid. The digests were incubated for 2 h at 37 °C, and a second 3.3 μL aliquot of trypsin was added for further overnight incubation at the same temperature. The digestion was stopped by the addition of 10 μL of trifluoroacetic acid, 10% v/v, and the samples were submitted to mass spectrometry analysis.

Fluorometric Assay. Inhibition of HCMV protease by β-lactam **2** was measured using the fluorogenic substrate *N*-Acetyl-Tbg-Tbg-Asn-(NMe)$_2$-Ala-AMC.[27] Substrate hydrolysis was followed by monitoring the increase in fluorescence intensity resulting from the release of the 7-amino-4-methylcoumarin moiety (λ_{ex} = 370 nm, λ_{em} = 415 nm, slit width 4 nm). Assay buffer contained 50 mM Tris/HCl, pH 8, 0.5 M Na_2SO_4, 50 mM NaCl, 0.1 mM EDTA, 1 mM TCEP, 0.05% w/v casein and 3% v/v DMSO. The reactions were initiated by addition of 25 nM enzyme to 25 °C preequilibrated mixtures containing 3 μM substrate (0.2 K_M) and 6−30 μM β-lactam **2** (3−10 IC_{50}). Progress curves in the presence of compound **2** were monitored for 50 min up to 30% substrate conversion (Figure 2).

Progress curves for the hydrolysis of **1** by HCMV protease were generated by monitoring the increase in fluorescence intensity resulting from the release of 4-methylumbelliferone (λ_{ex} = 395 nm, λ_{em} = 446 nm, slit width 4 nm). Assay buffer was as described above but without casein. The enzymatic reactions were initiated by addition of 60 μL of **1** (from 33.3 μM to 6.7 mM stocks in DMSO) directly into a 25 °C preequilibrated stirred mixture of 1.74 of mL assay buffer and 200 μL of HCMV protease (from 30 or 60 μM stocks in assay buffer as determined by the UV method described above). Data collection was started immediately for a period of 45 min (Figure 6A,B). Final concentrations of substrate were 1−200 μM and of enzyme were 3−6 μM. Similar buffer conditions were used for the stopped-flow experiments except that identical volumes (100 μL each) of enzyme and freshly prepared substrate in assay buffer were added simultaneously to the microcuvette (SFA-20, HI-TECH Scientific). The final enzyme concentration in these experiments was 3 μM (UV205/280 method). Final initial rates of the stopped-flow runs were based on the average of five repeats (Figure 7). A calibration curve was constructed with 4-methylumbelliferone and used for the conversion of fluorescence units into product concentration.

Chemical Hydrolysis of β-Lactam 1. The rate of chemical hydrolysis of compound **1** in assay buffer was determined as described above by monitoring the increase in fluorescence intensity as a function of time in the absence of HCMV protease. Initial rates of hydrolysis were obtained and plotted against the concentration of **1**. The slope of this linear plot gave $k_{hydr} = 9.7 \times 10^{-5}$ s^{-1} (Figure 5). The rate in the presence of DFP-inactivated HCMV protease was also measured and found to be similar to the rate in the absence of enzyme. The solubility of compound **1** in assay buffer was confirmed up to 200 μM by the linearity of absorbance readings at 315 and 330 nm. Precipitation was observed at 400 μM. All measurements were performed in duplicate.

4*S*-(4-Methylumbelliferone)-3*R*-methylazetidin-2-one-1-carboxylic Acid (4-Methylpyridyl) Amide (1). To a solution of 4-methylumbelliferone (236 mg, 1.34 mmol) in acetone (6 mL) at room temperature was added a 2.5 M solution of NaOH (540 μL, 1.34 mmol). The resulting mixture was stirred 2 min at room temperature, and then 3*R*-4-acetoxyazetidin-2-one[30] (295 mg, 2.06 mmol) in acetone (1 mL) was added. The resulting mixture was stirred for 40 h at room temperature and then concentrated in vacuo. The mixture was poured in 20 mL of 1 M HCl/brine (1:1) and extracted with EtOAc (2 × 30 mL). The combined organic phases were dried on $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (1:1 EtOAc/hex), yielding 4*S*-(4-methylumbelliferone)-3*R*-methylazetidin-2-one as a white solid (121 mg, 35%; the less polar diastereoisomer): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (d, J = 8.9 Hz, 1H), 6.84 (dd, J = 2.6, 8.6 Hz, 1H), 6.75 (d, J = 2.6 Hz, 1H), 6.52 (br s, 1H); 6.19 (d, J = 2.0 Hz, 1H); 5.38 (d, J = 1.0 Hz, 1H), 3.33 (q, J = 7.6 Hz, 1H), 2.40 (s, 3H), 1.48 (d, J = 7.6 Hz, 3H). A solution of the above 4*S*-(4-methylumbelliferone)-3*R*-methylazetidin-2-one (119 mg, 0.46 mmol), pyridin-4-yl-methylcarbamic acid phenyl ester[10] (136 mg, 0.60 mmol), triethylamine (130 μL, 0.92 mmol), and tributylphosphine (4 drops) was stirred in 1,2-dichloroethane (10 mL) at 70 °C for 4.5 h. The resulting mixture was concentrated under vacuum. The residue was purified by flash chromatography with EtOH/EtOAc (1:99) and by preparative TLC (4% EtOH/$CHCl_3$; 2 elutions). Compound **1** was obtained as a white solid (35 mg, 19%): HPLC (reverse phase) 100%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, J = 5.4 Hz, 2H), 7.56 (d, J = 8.5 Hz, 1H), 7.23 (d, J = 6.0 Hz, 1H), 7.11 (dd, J = 2.5, 8.9 Hz, 1H), 7.03 (d, J = 2.5 Hz, 1H), 6.96 (t, J = 6.0 Hz, 1H), 6.20 (d, J = 1.3 Hz, 1H), 5.76 (d, J = 1.6 Hz, 1H), 4.50 (d, J = 6.4 Hz, 2H), 3.44 (q, J = 7.6 Hz, 1H), 2.40 (d, J = 1.0 Hz, 3H), 1.52

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(d, *J* = 7.6 Hz, 3H); MS (FAB) 394 (M + H). HRMS; (M + H) calcd for $C_{21}H_{20}N_3O_5$ 394.1403, found 394.1389.

(*S*)-Benzylazetidin-2-one-1-carboxylic Acid 4-Azido-2,3,5,6-tetrafluorobenzylamide (5). To a solution of 4*S*-benzylazetidin-2-one[10] (151 mg, 0.94 mmol) in THF (5 mL) at −78 °C was added LiHMDS (0.96 mL, 0.98 mmol). After stirring at that temperature for 35 min a solution of (4-azido-2,3,5,6-tetrafluorobenzyl)carbamic acid phenyl ester (303 mg, 1.03 mmol) [prepared from 4-azido-2,3,5,6-tetrafluorobenzylamine[48] according to the method described by Thavonakham[49]] in THF (5 mL) was quickly added via syringe. The resulting solution was stirred for 10 min and then quenched by the addition of saturated NH_4Cl. Extraction with EtOAc was followed by washing with brine and drying over $MgSO_4$. Flash chromatography (20% EtOAc in hexanes) followed by preparative TLC (CH_2Cl_2) afforded compound **5** as a colorless oil (11 mg, 3%): ^1H NMR (400 MHz, $CDCl_3$) 7.33−7.24 (m, 3H), 7.17−7.15 (m, 2H), 6.97 (t, *J* = 5.4 Hz, 1H), 4.65−4.53 (m, 2H), 4.31−4.26 (m, 1H), 3.48 (dd, *J* = 13.8, 3.2 Hz, 1H), 2.99 (dd, *J* = 16.0, 5.7 Hz, 1H), 2.92 (dd, *J* = 13.8, 8.6 Hz, 1H), 2.74 (dd, *J* = 16.0, 3.2 Hz, 1H); IR (neat) 3362, 2123, 1766, 1705 cm^{-1}; MS (ES+) 430 $(M + Na)^+$, (ES−) 406 $(M - H)^-$; HRMS calcd for $C_{18}H_{14}F_4N_5O_2$ 408.1084, found 408.1092.

Compounds **2** and **3** were synthesized according to previously described procedures for closely related β-lactams.[10] Only the NMR and MS spectral data are provided.

Compound 2: HPLC 97%; ^1H NMR ($CDCl_3$) δ 8.58 (d, *J* = 5.4 Hz, 2H), 7.33−7.24 (m, 5H), 7.18 (d, *J* = 6.0 Hz, 2H), 6.98 (d, *J* = 8.3 Hz, 1H), 4.81 (m, 1H), 3.86 (ddd, *J* = 11.8, 1.5, 1.5 Hz, 1H), 3.48 (dd, *J* = 13.7, 3.2 Hz, 1H), 3.01−2.88 (m, 2H), 1.88−1.82 (m, 2H), 1.14 (d, *J* = 7.3 Hz, 3H), 0.98 (t, *J* = 7.3 Hz, 3H); FAB MS *m/z* 338 (MH^+).

Compound 3: HPLC 100%; ^1H NMR ($CDCl_3$) δ 8.59 (dd, *J* = 4.4, 1.6 Hz, 2H), 7.55−6.91 (m, 7H), 7.03 (ddd, *J* = 92.2 Hz, 6.4, 6.2 Hz, 1H), 4.56−4.46 (m, 2.5 H), 4.17−4.12 (m, 0.5H), 3.46 (dm, *J* = 129 Hz, 1H), 3.20−3.10 (m, 0.5H), 3.10−3.00 (m, 1H), 2.90−2.73 (m, 1.5 H); FAB MS *m/z* 307 (MH^+).

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Supporting Information Available: Derivation of the kinetic equations used in the numerical treatment of the data and a rapid protocol for the active site titration of HCMV protease using compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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