Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 4081–4088
Bioorganic & Medicinal Chemistry

Substrate variants versus transition state analogues as noncovalent, reversible enzyme inhibitors

Timothy P. Smyth*

Department of Chemical and Environmental Sciences, University of Limerick, National Technological Park, County Limerick, Ireland

Received 4 November 2003; accepted 25 May 2004
Available online 25 June 2004

Abstract—Reversible inhibitors are associated with fewer side effects than covalently binding ones and are, therefore, advantageous for treatment of conditions involving endogenous enzymes. Transition state analogue structures provide one design paradigm for such inhibitors; this paradigm seeks to exploit the capability of an enzyme active site to stabilise a transition state or associated intermediate. In contrast, structures that retain the functionality, and scissile bond of the substrate, can also act as reversible inhibitors; these are referred to here as substrate variants to distinguish them from substrate analogues. Their mode of inhibition depends on destabilisation of a reaction-path transition state or states. As the mode of destabilisation can be quite varied the scope to exploit substrate variants as reversible inhibitors is substantial. The two design paradigms are contrasted here and the case of substrate variants is delineated with a well-defined set of structures. These include the naturally occurring polypeptides BPTI (an inhibitor of a serine-based protease) and cathepsin propeptides (inhibitors of cysteine-based proteases) as well as the synthetic small-molecules cilastatin (an amide inhibitor of a zinc-based protease) and substituted mono- and tripeptides as inhibitors of cathepsins K and L.

1. Introduction

The design of enzyme inhibitors as structural analogues of intermediates, and as apparent analogues of transition states, is well established. The analogue design paradigm is based on replacing the main substrate functional group so as to entirely alter the reacting system, for example, substitution of a scissile bond by a nonscissile one. This allows incorporation of features into a stable molecule that mimic primary transition–state interactions of the natural substrate and the enzyme active site.[1] This approach, thus, seeks to exploit the capability of an enzyme active site to stabilise a transition state structure so that the stable transition state analogue is bound with a far higher affinity than the normal substrate. A generalised energy profile of this mode of noncovalent inhibition is shown schematically in Figure 1(a): the substrate profile is representative of scission of an ester or an amide involving the formation and breakdown of a tetrahedral intermediate. The profiles are shown as intersecting (orthogonal for simplicity) as the 'stable' inhibitor cannot undergo a reaction comparable to that of the substrate; the inhibitor profile shown represents some possible reaction path but one with a very high activation energy. The structural connection between the two profiles—the intermediate on the substrate profile and the stable analogue structure on the inhibitor profile (E·A)—is shown by the dashed line.

In contrast to the above, structures that retain the functionality and the scissile bond of the substrate can also act as potent, noncovalent, reversible inhibitors. The term substrate variant is used here to identify $R'C(O)NHR''$ as a variant of RC(O)NHR (the substrate) and to distinguish it from $RC(O)CH_2R$, which is an analogue of the substrate (see Table 1). A generalised energy profile that shows substrate variants acting as inhibitors is given in Figure 1(b). The profiles of the substrate and variant are shown as being parallel as both structures can, potentially, undergo the same type of transformation. Transfer of the energy surface for a substrate from an aqueous to an enzyme phase results in greater stabilisation of the transition state over that of the substrate (the default scenario for catalysis), whilst for the closely related structure Sv the corresponding transfer results in greater stabilisation of Sv over its cognate transition state (there is conservation of interaction energy when integrated over the energy

* Tel.: +353-61-202162; fax: +353-61-202568; e-mail: timothy.smyth@ul.ie

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.05.041

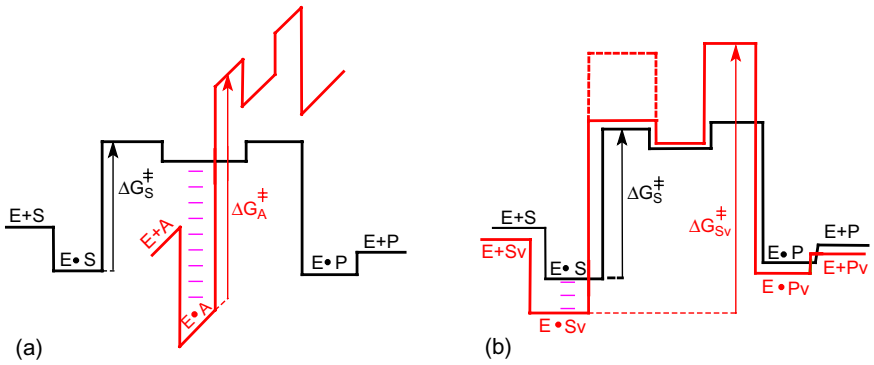


Figure 1. Schematic representation of the relationship between the energy profiles of (a) a substrate (S) versus that of a structure that is an analogue (A) of an intermediate or transition state as an inhibitor and (b) a substrate (S) versus that of a substrate variant (Sv) as an inhibitor.

Table 1. Protease inhibitor characteristics of substrate variants, transition state analogues and substrate analogues

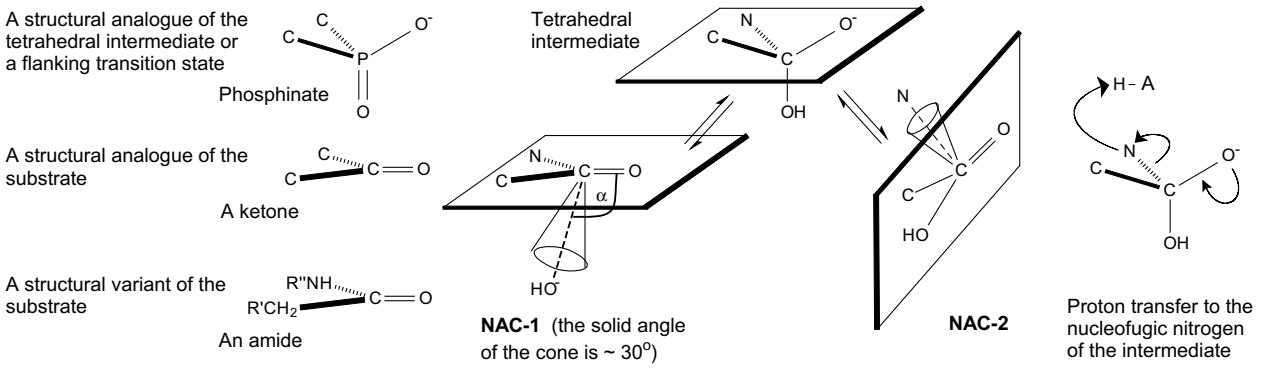
	Substrate variants	Transition state analogues	Substrate analogues
Functionality presented at the active site	Amide bond of unaltered reactivity compared with normal substrate	Various nonreactive groups	Carbonyl (or related) group with enhanced reactivity over normal substrate
Design paradigm—binding versus reactivity	Binding exclusively; normal reaction pathway is destabilised within the active site; noncovalent interaction primarily	Binding exclusively; stable structure—possible reaction pathways are inherently destabilised; noncovalent interaction	Binding and reactivity;[a] mechanism-based covalent interaction
Examples	Endogenous inhibitors: BPTI and related structures; various propeptides	Endogenous inhibitors: pepstatin.[b]	Exogenous inhibitors: β-lactams (both naturally occuring and synthetic); γ-lactams with an EWG on nitrogen;[c] lactones; ketones; aldehydes etc.
	Exogeneous inhibitors: cilastatin; peptides A and B (see Chart 2 here)	Exogenous inhibitors: phosphinates; statines, norstatine;[b] etc.	

[a] The relevance of reactivity in acylation-type inhibitors has been reviewed: Ref. 21.
[b] Ref. 22; for comments on these as strict transition state analogues see: Ref. 23.
[c] Ref. 24.

surface as enzymes provide an alternative medium for a reaction but are not net energy producers). It follows that Sv will act as a competitive, reversible inhibitor in a reaction where S is the substrate. The effectiveness of Sv as an inhibitor, thus, depends on destabilisation of a reaction-path transition state or states (large $\Delta G^{\#}_{Sv}$) combined with tighter binding of the variant (E·Sv) compared with the normal substrate. This mode of inhibition is less well established compared with that of transition state analogues and it is instructive to delineate the variety of inhibitory modes of a well-defined set of such structures. The examples dealt with here include the naturally occurring polypeptides BPTI (an inhibitor of a serine-based protease) and cathepsin propeptides (inhibitors of cysteine-based proteases) as well as the synthetic small-molecules cilastatin (an amide inhibitor of a zinc-based protease) and substituted mono- and tripeptides as inhibitors of cathepsins K and L. The mode of destabilisation is distinct in each case, which indicates that the scope to exploit substrate variants as reversible inhibitors is substantial.

2. Discussion

Base-catalysed hydrolysis of an amide bond occurs in two steps involving formation and fragmentation of the intermediate resulting from hydroxide addition to the amide carbonyl (Scheme 1). The steps of hydroxide addition and amine departure are viewed as proceeding along a Bürgi–Dunitz trajectory of nucleophile/nucleofuge addition/release to/from the carbonyl group/cognate intermediate.[2] The reaction ensemble wherein the nucleophile/nucleofuge is appropriately juxtaposed, in terms of separation (2.7–3.0 Å) and angle of approach/departure ($\alpha \sim 103 \pm 15°$), to initiate bond formation/complete bond scission, has been labelled as a near-attack conformer[3] (NAC-1 and NAC-2 in Scheme 1). The same features apply to the general base-catalysed addition of a serine alkoxide or cysteine thiolate (for these proteases a second step is required to effect hydrolysis of the acylated active site). Bond formation and scission involves heavy-atom displacement along specific reaction coordinates and these processes dynamically change the values of nonbonded interactions between substrate components and active-site residues. These interactions, which are acutely (and anharmonically) dependent on internuclear separation, play a central role in substrate binding and in transition state stabilisation. In addition to these heavy atom displacements, proton transfer from an appropriately juxtaposed acid to the nucleofugic nitrogen of the tetrahedral intermediate is essential (Scheme 1). The structural requirements of substrate specificity and efficiency of catalysis must involve a



Scheme 1.

balance between the active-site ground-state dimensions with the extent and dynamics of their elasticity.[4] This provides a basis for understanding features that are common to the inhibitory mode of action of the very distinct structures, cilastatin, BPTI, cathepsin propeptides and small-molecule inhibitors of cathepsins.

2.1. Cilastatin

Cilastatin (Chart 1) was developed empirically as a reversible inhibitor of membrane dipeptidase (MDP), which is a zinc-based mammalian enzyme. This enzyme catalyses hydrolysis of the cysteinylglycine dipeptide end group of leukotriene-D_4 (LTD_4, Chart 1), amongst other metabolic processes, and it also cleaves the β-lactam ring of the antibiotic imipenem. This latter problem is overcome by co-administration of cilastatin with imipenem.[5] A detailed analysis of the X-ray structure of MDP with cilastatin bound at its active site[6] together with the results of docking studies of substrate molecules, led to the depiction of its mode of action as (one form of) a substrate variant.[7]

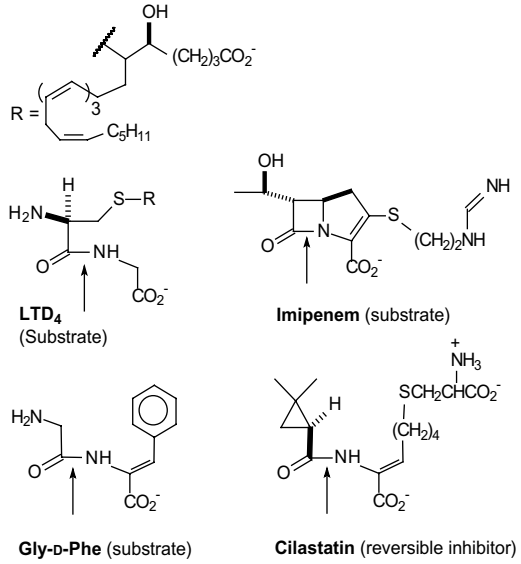


Chart 1. Substrate and inhibitor structures of membrane dipeptidase (MDP). The scissile amide bond is indicated (arrow).

The active site of MDP is a rectangular pocket on the protein surface measuring approximately 10 Å (length) × 6 Å (width) × 6 Å (depth) and its substrate specificity is, largely, defined by these dimensions.[6] Key internuclear separations of cilastatin bound at the active site are shown in Figure 2. The view is from the open top face of the active site towards the base, which contains two zinc ions and a water molecule; relevant internuclear separations of these are given in Figure 3. The carboxylate of Asp288 (not shown) is ideally positioned to provide general-base/acid catalysis of proton relay from the water molecule and subsequent transfer to the nucleofugic nitrogen of cilastatin. The zinc-bound water is in a perfect NAC for formation of an addition intermediate: the oxygen atom is at 2.82 Å from the amide carbonyl and forms an angle, α, of 102.9°. In addition, the imidazole ring of His152 is positioned so as to form a good hydrogen bond with the oxyanion of this intermediate. These features are ideally suited for addition of the hydroxide ion to generate an addition intermediate—there is no impediment to progress along this reaction coordinate. The reaction coordinate for departure of the amine leaving group from this intermediate is along the Tyr68-to-Tyr252 axis (Fig. 2). The dimethylcyclopropyl group and the alkyl side chain of cilastatin lie along this axis and clearly buttress the side chains of these peripheral tyrosines. At a separation of 3.52 Å the interaction energy of a cyclopropyl carbon with that of Tyr68 is only very marginally stabilising, whereas, at a separation of 3.90 Å the interaction energy of an alkyl side-chain carbon with that of Tyr252 is just about optimal (see Fig. 4 and associated equation[8]). Overall, these interactions contribute to the binding of cilastatin as a NAC at the active site; note that K_i for cilastatin is 0.1 μM,[5b] which contrasts with a K_m value of 1 mM for a typical (synthetic) substrate—glycyldehydrophenylalanine (Gly-D-Phe; Chart 1).[9] Departure of the amine leaving group from the intermediate derived from cilastatin requires an internuclear separation of the amine nitrogen and the carbonyl carbon of 2.7–3.0 Å, that is the separation occurring in the product NAC (NAC-2 in Scheme 1). As the amide bond length in cilastatin is 1.38 Å a heavy-atom displacement of 1.32–1.62 Å is necessary. Such a displacement would place the dimethylcyclopropyl and alkyl end group of cilastatin at internuclear separations to Tyr68 and Tyr252, respectively, that would be highly destabilising. Reducing *R*

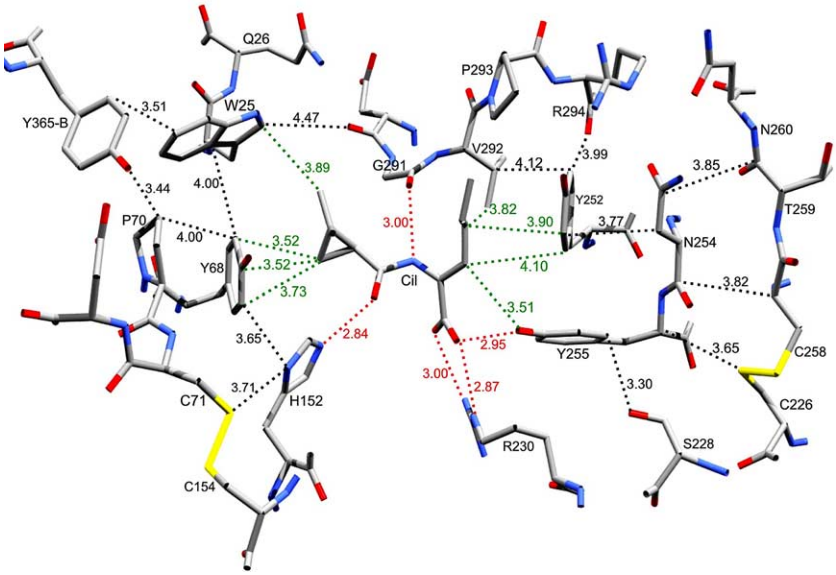


Figure 2. Heavy-atom separations (Å) of cilastatin with MDP active-site residues (PDB code 1ITU).[6] Hydrogen bonds to cilastatin are shown in red and nonpolar interactions in green; other selected distances are shown in black. Note that the tyrosine in the top left here, Y365-B, is part of the (disulfide) linked second monomer unit of MDP.

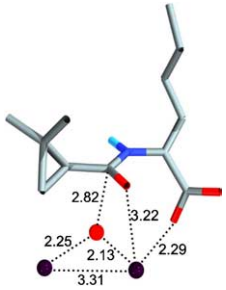


Figure 3. Heavy-atom separations (Å) of cilastatin with Zn^{2+} (black) and water/hydroxide (red). A polar hydrogen only has been added to cilastatin here.

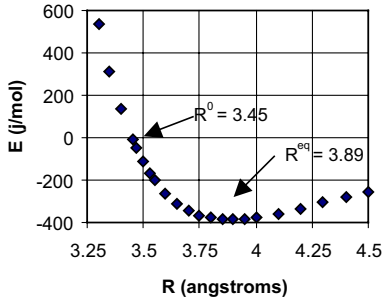


Figure 4. Potential energy E (J/mol) versus internuclear separation R for C/C nonbonded interaction.[8]

for one C/C nonbonded atom pair from the R^{eq} value of 3.89 to 2.57 Å (a reduction of 1.32 Å) would give an interaction energy of +21.90 kJ/mol whilst reduction by 1.62 to 2.27 Å would give a value of +68.13 kJ/mol (see Fig. 4 and associated equation).[10] Some of the dynamic motions of enzyme residues—depending on their time scale[11]—can attenuate these kinds of interactions, however, there must be a limit on the capacity of the active site to readily expand beyond that for which it has evolved in terms of its substrate set, in particular, for an enzyme that has a stringent substrate specificity.[4] This appears to be the case with MDP. The active site dimensions in native and cilastatin-bound MDP are very similar; this and other data on substrate specificity point to a limited capability of the MDP active site to readily expand much beyond the dimensions of the native protein[7] (the tight fit of cilastatin along the Tyr68-to-Tyr252 axis may serve to restrain antisymmetric motion (expansion) of the active site along this axis[4b]). Based on the internuclear separations available from the X-ray structure of cilastatin-bound MDP (Fig. 2) it was estimated that an expansion of 0.52 Å could be accommodated,[7] giving an internuclear separation of the amine nitrogen and carbonyl carbon of 1.90 Å, whereas, attaining the minimum required separation of 2.7 Å would require a significant further energy input. In contrast, with the substrates LTD_4 and Gly-D-Phe this minimum separation could be readily accommodated within the active site dimensions. Thus, docking of these showed that the scissile amide bond of each could bind in an NAC orientation at the active site in a manner essentially identical to that observed for cilastatin. The docking studies showed that there is over 1 Å more available for displacement of the amine leaving group along the horizontal axis for each of these compared to that available to cilastatin.[7] An increase of 1.52 Å between the amine nitrogen and carbonyl carbon of LTD_4 (giving a total separation of approximately 2.90 Å) occurring uniquely by displacement of the glycl moiety toward Tyr252 would still result in a favourable interaction energy of the corresponding nonbonded carbons. Whatever the degree of flexibility of the peripheral residues that facilitates this displacement for a normal substrate, it is apparent that this is not sufficient to accommodate the same process for cilastatin.

The key features of cilastatin as a substrate variant can be summarised as follows: it binds using the same noncovalent interactions as the primary functional group of the substrate, but in addition, its substituent array buttresses residues on the periphery of the active site at internuclear separations that are close to the limit for favourable interaction energies—it is tight fitting and by default tight binding; this arrangement impedes heavy-atom displacement along a key reaction coordinate axis thereby destabilising the associated transition state. It fits the profile shown for a substrate variant (Sv) in Figure 1(b) wherein the step of high activation energy is associated with breakdown of the tetrahedral intermediate.

2.2. BPTI

BPTI is a naturally occurring 58 unit polypeptide that is a potent ($K_i = 10^{-13}$ M), endogenous, inhibitor of trypsin—a serine-based protease.[12] Enzyme-catalysed conversion to products does not occur—the rate of hydrolysis of BPTI by trypsin ($k_{cat} = 8.7 \times 10^{-10}\ s^{-1}$) is slower than that of a typical substrate by a factor of 10^{11}. Crystal structure data show that BPTI—and a number of related protease inhibitors[12b–e]—bind at the active site with the scissile amide bond positioned in a catalytically competent (NAC), substrate-like manner. A typical example is given in Figure 5:[12f] this shows an internuclear separation of 2.78 Å between the side chain oxygen of the active-site Ser195 to the carbon of the scissile amide carbonyl (at an angle $\alpha = 89.4°$) and, in addition, Gly193 and Ser195 are suitably positioned to provide hydrogen-bond stabilisation of the tetrahedral intermediate derived from addition of the Ser195 alkoxide to the Lys15-BPTI carbonyl. His57 is suitably placed to provide general-base/acid catalysis of proton relay from Ser195 and subsequent transfer to the nucleofugic nitrogen of Ala16. A detailed analysis of the interaction of BPTI, and of a substrate, with trypsin has been carried out by Peräkylä and Kollman using a quantum mechanical-free energy (QM-FE) approach together with molecular dynamic simulations.[13a] These calculations showed that the intermediate generated by addition of the alkoxide of Ser195 to Lys15-BPTI, and the transition states associated with its formation and fragmentation, are destabilised with respect to the corresponding entities generated from a substrate structure. The exclusion of water by BPTI from the active site (Fig. 5 (i)) plays a major role in this destabilisation as does the less favourable interaction energies of trypsin and BPTI amino acid residues. On this basis a decrease in catalytic rate of 10^6–10^9 (of a total reduction of 10^{11}) was accounted for. The remaining decrease of 10^5–10^2 was attributed to restricted diffusion of the cleaved amino moiety, which leads to reformation of the scissile amide bond in preference to hydrolysis of the acylated active site (the restricted diffusion of the leaving group is due, partly, to an intramolecular constraint as BPTI is crosslinked with disulfide bonds). Progress along the hydrolysis reaction coordinate can also be impeded by exclusion of water. Thus, the associated transition state(s) may be destabilised due to the absence of solvent water molecules, and/or access of the required nucleophilic water molecule may be blocked; this latter process on its own will reduce the rate of the hydrolytic step irrespective of any destabilisation of the transition state(s).

The key features of BPTI as a substrate variant can be summarised as follows: it binds in a catalytically competent (NAC), substrate-like manner; cleavage of the amide bond to generate the acylated enzyme occurs slowly as the intermediate and associated transition states are destabilised within the enzyme environment; religation of the cleaved amide bond is thermodynamically favourable, and this occurs more readily than hydrolysis of the acylated enzyme as water is excluded from the active site.[13] These observations are delineated in the

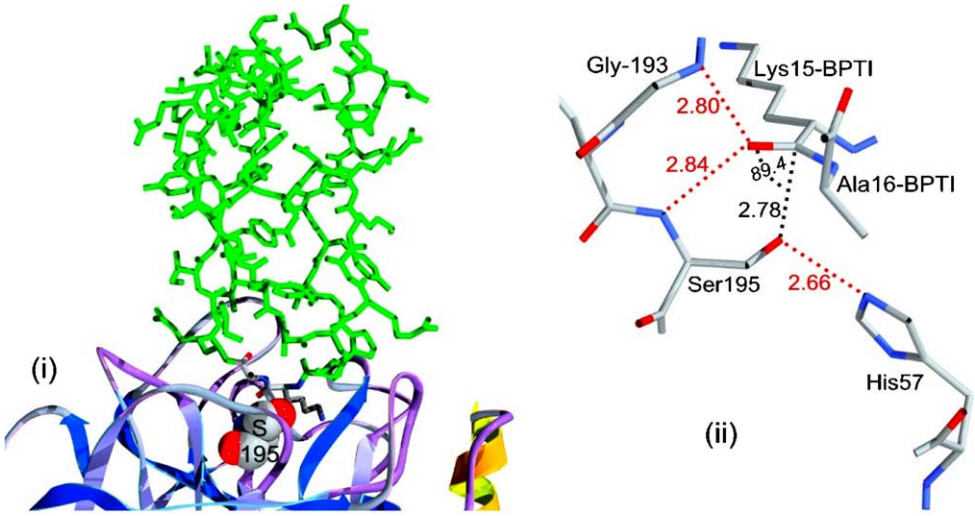


Figure 5. (i) BPTI (in green except for Lys15-Ala16) bound to trypsin (PDB code 3TGI).[12f] The enzyme is shown as a ribbon diagram with the active-site serine highlighted. (ii) Internuclear separations (Å) of active-site components; Lys15 and Ala16 occupy the S1 and S1′ binding sites, respectively. Hydrogen bonding interactions are shown in red.

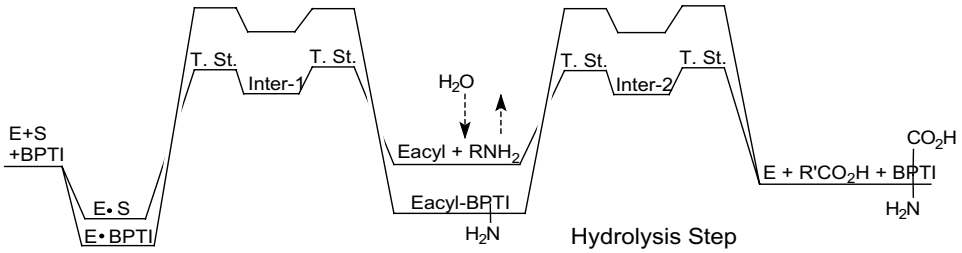


Figure 6. Schematic energy profile of trypsin-catalysed hydrolysis of a normal polypeptide substrate (S), and with BPTI. Adapted from Ref. 13a.

schematic reaction profile diagram shown in Figure 6: occupation of the energy minima—E·BPTI and/or Eacyl-BPTI-NH_2—provides equally effective inhibition.[14]

2.3. Cathepsins

Cathepsins are cysteine-based proteases, some of which have been identified as important targets for inhibition.[15] The cathepsins are synthesised in an inactive proenzyme form bearing a 60–100 unit polypeptide (the propeptide) that is tightly bound (noncovalently) at the active site; in the case of cathepsin-L the 96 unit propeptide has a K_i value of 0.088 nM.[15a] It has been established that the propeptide binds in a reverse-substrate mode, that is the amino-to-carboxy-terminus alignment is opposite to that of a substrate molecule and this has implications regarding catalysis of cleavage of the scissile bond. The amino acid substituents, that are attached to either side of the scissile amide bond, occupy the various binding pockets (...S2.S1.S1′.S2′...), and generate favourable interaction energies that contribute to the observed tight binding.[16] Crystal structure data show that, although the scissile amide bond is held in proximity to the active-site cysteine, the reverse-substrate binding precludes its juxtaposition as a catalytically competent NAC. The crystal structure of procathepsin-K[16a] (Fig. 7, (i) and (ii)) shows the sulfur of the active-site cysteine (Cys124) at 3.76 Å from the propeptide carbonyl, Thr76-p, and at 4.11 Å from the imidazole ring of His261 (the general-base/acid). A simple rotation of the cysteine side chain places the sulfur atom in a more reactant-like orientation (Fig. 7 (iii)), however, the key discrepancy remains the remote location of the nucleofugic nitrogen (#) from the imidazole of His261 that catalyses the proton relay process. A normal substrate would have its nucleofugic nitrogen close to the position occupied by

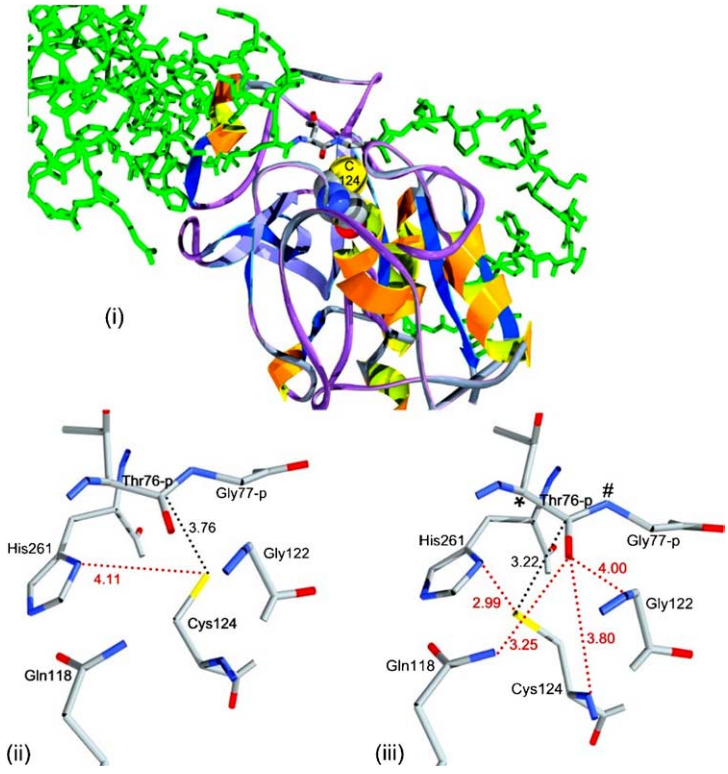


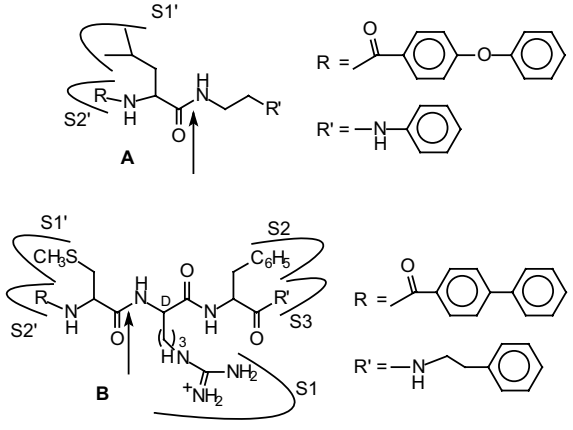
Figure 7. (i) Procathepsin-K (PDB code 1BY8).[16a] The enzyme is shown as a ribbon diagram with the active-site cysteine highlighted. The propeptide is shown in green except for Thr76-Gly77. (ii) Active-site internuclear separations (Å) from the crystal structure, and (iii) as (ii) but showing a rotamer of the side chain of Cys124. Hydrogen bonding interactions are shown in red.

the labelled carbon atom (*) of the propeptide, which is appropriate for facile completion of the proton relay. The imidazole of His261 is not solvent accessible with the propeptide in place, so a water mediated proton relay cannot occur (unlike the case of BPTI, water is not totally excluded from contact with the propeptide residues at the active site). In the case of the propeptide, therefore, the transition state leading from the addition intermediate to the acylated enzyme is destabilised; the experimental data indicate that the extent of this destabilisation is sufficient to preclude scission of the propeptide.

Recently, the synthetic small molecules **A**[15c] and **B**[15d] (Chart 2) were developed as selective, high affinity and reversible inhibitors of cathepsins K and L, respectively. X-ray data showed that **B** was bound as shown in Chart 2, which mirrors the reverse-substrate orientation of the propeptide on which it was modelled.[15d] Given that the specificity pockets of cathepsins K (PDB code 1BY8)[16a] and L (PDB code 1CS8)[16b] are quite similar, the selectivity shown by **A** and **B** is significant. These small molecules, together with their cognate propeptides, constitute another form of substrate variant as enzyme inhibitor.

3. Conclusion and perspectives

The common inhibitory feature of the diverse structures presented in the foregoing sections, is the high affinity of the substrate variant in its ground state for the active site combined with destabilisation of a key reaction-path transition state. The mode of this destabilisation is distinct in each case, which indicates that the scope to exploit substrate variants as inhibitors may be substantial. Reversible inhibitors show fewer side effects compared to covalently binding inhibitors, whilst, substrate-based structures can be quite discriminating amongst closely related members of a family of enzymes (as per **A** and **B** above). These are desirable inhibitor characteristics for treatment of conditions involving endogenous enzymes. The explicit design of substrate variants has the potential to expand the base of such inhibitors. Cilastatin is a prime functional example with a positive safety and toxicity record in clinical usage.[5b,17] Binding of a sufficiently high affinity for therapeutic use is not the preserve of small-molecule transition state analogues or macromolecular polypeptides, and neither are the characteristics of bioavailability and stability limited to nonsubstrate-type structures.[15b]



	K_i(μM)	
	Cathepsin-K	Cathepsin-L
A	0.006	> 10
B	5.9	0.019

Chart 2. The substituted peptide (**A**) and tripeptide (**B**) that act as reversible inhibitors of cathepsins K and L, respectively.[15c,d] The scissile amide bond is indicated (arrow). The binding pockets are identified for **B** from the crystal data,[15d] whereas, those for **A** are assigned here by analogy with **B**.

Cilastatin and peptide **A** were developed, largely, on an empirical basis from a lead structure and, subsequently, using a relatively small library of structures for optimisation. With tripeptide **B**, modelling of the propeptide region and docking studies were key components of the development strategy; optimisation also required only a relatively small structure library. The BPTI design paradigm may be less readily exploited compared to that embodied in cilastatin and in the peptides **A** and **B**. Penicillin and related structures provide, however, a relevant small-molecule parallel. Inhibition of bacterial (serine-based) peptidases by β-lactams involves their binding in a substrate-like NAC followed by amide bond cleavage yielding an acylated enzyme and restricted diffusion of the cleaved amino group—this is covalently linked to the acylated active site. The retained amino group impedes access by a water molecule, thereby, retarding hydrolysis of the acylated enzyme. Although the cleaved amino group may be appropriately juxtaposed[18]—as a product NAC—to reform the β-lactam bond, this is not thermodynamically favourable and, so, the mode of inhibition is essentially irreversible. Formation of a lactam bond has been shown to occur at an acylated active site, however, to generate (larger-ring) γ-lactams.[19a,b] In the context of using these to develop irreversible (covalent) inhibitors of bacterial peptidases this finding is negative,[19c] however, the γ-lactam structure type represents a rudimentary, small-molecule embodiment of the BPTI design paradigm.

Substrate variants may also have application as inhibitors of rapidly evolving microorganisms or cell lines. A detailed analysis of the variability of amino acid residues of the protease enzyme produced by HIV-virus strains known to be (clinically) resistant to a number of distinct (reversible) protease inhibitors showed that,[20] (i) active-site residues that had favourable interaction energies with the substrate were highly conserved (i.e., were subject to low mutation rates), and (ii) that many resistant strains showed a single amino acid change only. Significantly, the residue mutated (in the resistant strain) strongly interacted originally (in the wild type) with a fragment of an inhibitor structure but only very weakly, if at all, with the substrate. The clear inference[20] was that use of inhibitor structures designed to maximise interaction with highly conserved active site

residues—those involved in substrate binding *and transformation*—should reduce the scope for evolution of resistant strains. Some of the design features of substrate variants described in the preceding sections could lead to structures that meet these requirements.

Acknowledgements

I am grateful to Dr. J. Gerard Wall for helpful discussions.

References and notes

1. (a) Wolfenden, R. *Bioorg. Med. Chem.* **1999**, *7*, 647–652, and references cited therein; (b) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305–341; (c) Brik, A.; Wong, C.-W. *Org. Biomol. Chem.* **2003**, *1*, 5–14, and references cited therein.
2. (a) Bürgi, H. B.; Dunitz, J. D.; Shefter, E. *J. Am. Chem. Soc.* **1973**, *95*, 5065–5067; (b) Shefter, E. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: NY, 1978; Chapter 9 and references cited therein; (c) Dunitz, J. D. *X-ray Analysis of Organic Molecules*; Cornell Univeristy Press: Ithaca, NY, 1979; Chapter 7.
3. (a) Lightstone, F. C.; Bruice, T. C. *J. Am. Chem. Soc.* **1996**, *118*, 2595–2605; (b) Bruice, T. C.; Benkovic, S. J. *Biochemistry* **2000**, *39*, 6267–6274.
4. For a view on how symmetric motion of the side walls of an S1 protease pocket may influence substrate specificity see: (a) Ota, N.; Agard, D. A. *Prot. Sci.* **2001**, *10*, 1403–1414; (b) Miller, D. W.; Agard, D. A. *J. Mol. Biol.* **1999**, *286*, 267–278.
5. (a) Kropp, H.; Sundelof, J. G.; Hajdu, R.; Kahan, F. M. *Antimicrob. Agents Chemother.* **1982**, *22*, 62–70; (b) Birnbaum, J.; Kahan, F. M.; Kropp, H.; Macdonald, J. S. *Am. J. Med.* **1985**, *78*(Suppl. 6A), 3–21, and references cited therein; (c) Graham, D. W.; Ashton, W. T.; Barash, L.; Brown, J. E.; Brown, R. D.; Canning, L. F.; Chen, A.; Springer, J. P.; Rogers, E. F. *J. Med. Chem.* **1987**, *30*, 1074–1090.
6. Nitanai, Y.; Satow, Y.; Adachi, H.; Tsujimoto, M. *J. Mol. Biol.* **2002**, *321*, 177–184.
7. Smyth, T. P.; Wall, J. G.; Nitanai, Y. *Bioorg. Med. Chem.* **2003**, *11*, 991–998.
8. Dunitz, J. D.; Gavezzotti, A. *Acc. Chem. Res.* **1999**, *32*, 677–684, $E\ (\mathrm{J/mol}) = (226145\exp(-3.47R) - 2418R^{-6})$.
9. Campbell, B. J. *Meth. Enzymol.* **1970**, *19*, 722–729.
10. Highly destabilising interaction energies generated by a given ΔR cannot be compensated for by establishing new contacts with other active site residues, due to the acute anharmonic nature of nonbonded atom–atom interactions.
11. Relatively large motions involving opening and closing of a protein hinge region or flap—associated with initial substrate binding and/or final product release—are relatively slow processes occurring on the millisecond timescale. See: (a) Hammes, G. G. *Biochemistry* **2002**, *41*, 8221–8228; (b) Osborne, M. J.; Schnell, J.; Benkovic, S. J.; Dyson, H. J.; Wright, P. E. *Biochemistry* **2001**, *40*, 9846–9859.
12. (a) Estell, D. A.; Wilson, K. A.; Laskowski, M., Jr. *Biochemistry* **1980**, *19*, 131–137, and references cited therein; (b) Rühlmann, A.; Kukla, D.; Schwager, P.; Bartels, K.; Huber, R. *J. Mol. Biol.* **1973**, *77*, 417–436; (c) Tschesche, H. *Angew. Chem., Int. Ed.* **1974**, *13*, 10–28; (d) Shaw, G. L.; Davis, B.; Keeler, J.; Fersht, A. R. *Biochemistry* **1995**, *34*, 2225–2233, and references cited therein; (e) Lapatto, R.; Krengel, U.; Schreuder, H. A.; Arkema, A.; de Boer, B.; Kalk, K. H.; Hol, W. G. J.; Grootenhuis, P. D. J.; Mulders, W. M.; Dijkema, R.; Theunissen, H. J. M.; Dijkstra, B. W. *EMBO J.* **1997**, *16*, 5151–5161; (f) Pasternak, A.; Ringe, D.; Hedstrom, L. *Prot. Sci.* **1999**, *8*, 253–258.
13. (a) Peräkylä, M.; Kollman, P. A. *J. Am. Chem. Soc.* **2000**, *122*, 3436–3444; (b) Radisky, E. S.; Koshland, D. E., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10316–10321.
14. (a) The equilibrium constant values for a wide variety of such naturally occurring polypeptide inhibitors have been measured and, in general, are close to unity: Ardelt, W.; Laskowski, M., Jr. *J. Mol. Biol.* **1991**, *220*, 1041–1053; (b) Laskowski, M., Jr.; Qasim, M. A. *Biochim. Biophys. Acta* **2000**, *1477*, 324–337.
15. (a) Coulombe, R.; Grochulski, P.; Sivaraman, J.; Ménard, R.; Mort, J. S.; Cygler, M. *EMBO J.* **1996**, *15*, 5492–5503; (b) Lecaille, F.; Cotton, J.; McKerrow, J. H.; Ferrer-Di Martino, M.; Boll-Bataillé, E.; Gauthier, F.; Lalmanach, G. *FEBS Lett.* **2001**, *507*, 362–366; (c) Altmann, E.; Renaud, J.; Green, J.; Farley, D.; Cutting, B.; Jahnke, W. *J. Med. Chem.* **2002**, *45*, 2352–2354; (d) Chowdhury, S. F.; Sivaraman, J.; Wang, J.; Devanathan, G.; Lachance, P.; Qi, H.; Ménard, R.; Lefebvre, J.; Konishi, Y.; Cygler, M.; Sulea, T.; Purisima, E. O. *J. Med. Chem.* **2002**, *45*, 5321–5329.
16. (a) Lalonde, J. M.; Zhao, B.; Janson, C. A.; D'Alessio, K.; Mcqueney, M. S.; Orsini, M.; Debouck, C.; Smith, W. W. *Biochemistry* **1999**, *38*, 862–869; (b) Groves, M. R.; Coulombe, R.; Jenkins, J.; Cygler, M. *Proteins: Struct. Funct. Genet.* **1998**, *32*, 504–514.
17. (a) Pavesio, D.; Pecco, P.; Giacchino, M.; Dotti, M. *J. Chemother.* **1991**, *3*(suppl 1), 218–221; (b) Biglino, A.; Bonasso, M.; Gioannini, P. *J. Chemother.* **1991**, *3*(suppl 1), 208–212; (c) Feldman, C.; White, H.; O'Grady, J.; Flitcroft, A.; Briggs, A.; Richards, G. *Int. J. Antimicrob. Agents* **2001**, *17*, 177–188.
18. The crystal structure data of D-alanyl-D-alanine carboxypeptidase with the β-lactam-ring-cleaved structures of cephalothin and cefotaxime covalently linked to the active-site serine show the nitrogen of the cleaved amino leaving group positioned at 2.71 and 2.77 Å and at angles, α, of 90.8 and 114.2°, respectively, from the carbonyl of the acylated active site. See PDB codes 1CEG and 1CEF: Kuzin, A. P.; Liu, H.; Kelly, J. A.; Knox, J. R. *Biochemistry* **1995**, *34*, 9532–9540.
19. (a) Barker, C. V.; Korn, S. R.; Monteith, M.; Page, M. I. *Chem. Commun.* **1999**, 721–722; (b) Westwood, N. J.; Claridge, T. D. W.; Edward, P. N.; Schofield, C. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2973–2978; (c) Hadfield, P. S.; Casey, L. A.; Galt, R. H. B.; Vilanova, B.; Page, M. I. *Arkivoc* **2002**, 125–144.
20. Wang, W.; Kollman, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 114937–114942.
21. (a) Sykes, N. O.; Macdonald, S. J. F.; Page, M. I. *J. Med. Chem.* **2002**, *43*, 2850–2856; (b) Imming, P.; Klar, B.; Dix, D. *J. Med. Chem.* **2000**, *43*, 4328–4331.
22. Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305–341.
23. Rich, D. *J. Med. Chem.* **1985**, *28*, 264–273.
24. Macdonald, S. J. F. et al. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 243–246.