Structural Analysis of Spiro β-Lactone Proteasome Inhibitors

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Structural Analysis of Spiro β-Lactone Proteasome Inhibitors

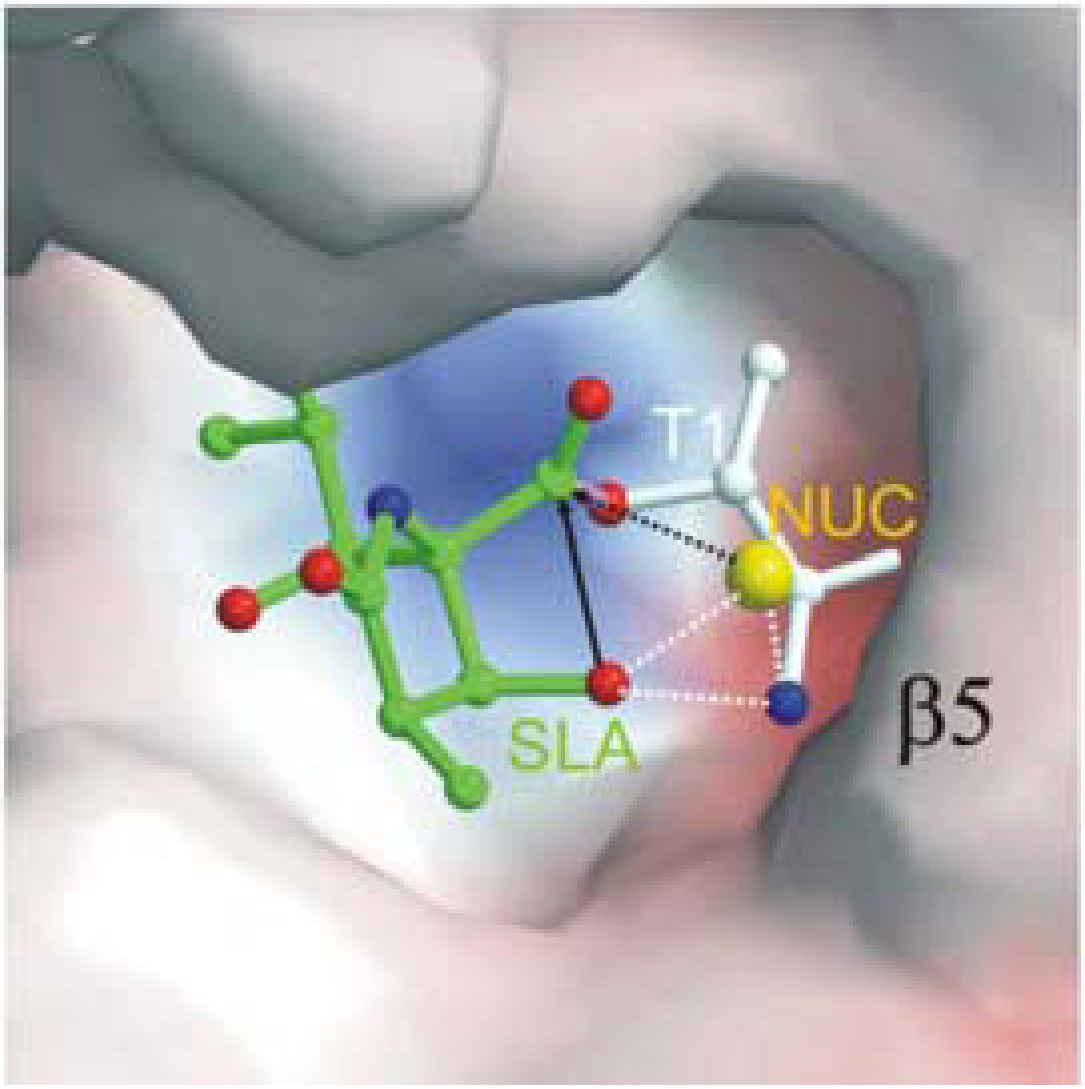
Michael Groll, **Emily P. Balskus**, and **Eric N. Jacobsen***
Contribution from the Center for Integrated Protein Science at the Department Chemie, Lehrstuhl für Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Germany, and the Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138.

Abstract

Email: jacobsen@chemistry.harvard.edu.

Protein Data Bank Accession Codes Coordinates have been deposited in the RCSB Protein Data Bank under the accession codes 3DY3 and 3DY4.

Supporting Information Available: Table of crystallographic data collection and refinement statistics, and experimental details of the attempted kinetic studies. This information is available free of charge via the internet at http://pubs.acs.org.



Spiro β-lactone-based proteasome inhibitors were discovered in the context of an asymmetric catalytic total synthesis of the natural product (+)-lactacystin (**1**). Lactone **4** was found to be a potent inhibitor of the 26S proteasome, while its C-6 epimer (**5**) displayed weak activity. Crystallographic studies of the two analogs covalently bound to the 20S proteasome permitted characterization of the important stabilizing interactions between each inhibitor and the proteasome's key catalytic *N*-terminal threonine residue. This structural data supports the hypothesis that the discrepancy in potency between **4** and **5** may be due to differences in the hydrolytic stabilities of the resulting acyl enzyme complexes.

The ubiquitin-proteasome pathway is responsible for the controlled protein degradation in cells through conjugation to ubiquitin and subsequent cleavage by a multimeric protein complex known as the 26S proteasome. This process is involved in regulating many essential biological events, including the destruction of misfolded and misassembled proteins, cell division, cell differentiation, and development of the immune response.[1]

Much knowledge of the proteasome's role in cellular function has been gained from the study of small-molecule inhibitors of the enzyme (Figure 1).[2] Lactacystin, the first natural product

to be identified as a proteasome inhibitor,[3] has been found to possess a mode of action involving covalent modification of the *N*-terminal threonine residue of subunit β5/X of the mammalian 20S proteasome.[4] Additional mechanistic in vitro and in vivo studies have served to establish the cis-fused β-lactone omuralide (**2**) as the relevant pharmacophore.[5] A similar mechanism of proteasome inhibition has been established for the salinosporamides, a structurally related family of natural products.[6]

The final steps of our recently-disclosed asymmetric catalytic total synthesis of lactacystin (**1**) utilized intermediates bearing an unusual spiro-fused β-lactone.[7] Several analogs bearing this functional group were assayed for their ability to inhibit the proteolytic activity of rabbit muscle 26S proteasome.[8] Spiro β-lactone **4** was found to inhibit all three proteolytic 20S subunits at levels similar to omuralide under identical conditions (83% inhibition of the β5 subunit at 10 μM). In contrast, C-6 epimeric spiro β-lactone **5** was inactive at concentrations below 200 μM. We have carried out fuller investigations of the binding of these epimeric compounds to the proteasome, with the hope that elucidating the basis for their difference in activity might provide mechanistic insight into proteasome inhibition. Here we report structural evidence that the spiro β-lactones function by acylation of the hydroxyl group of the *N*-terminal threonine, and suggest that the potency of these proteasome inhibitors is linked to the hydrolytic stability of the acyl-enzyme intermediates.

X-ray crystallographic studies of complexes of the 20S proteasome with small molecule inhibitors have provided valuable information about these important compounds and their modes of action,[9] and both omuralide and salinosporamide have been characterized in this manner.[10,11] As an initial step towards understanding the behavior of the spiro β-lactones in the presence of proteasome, we obtained crystal structures of their respective acyl-enzyme intermediates. Lactones **4** and **5** were co-crystallized with the yeast 20S proteasome by soaking single proteasome crystals with inhibitor for 60 minutes at a final concentration of 5 mM. Crystallographic refinement started from the coordinates of the yeast 20S proteasome,[10] followed by anisotropic overall temperature-factor correction and positional refinement using CNS[12] and cyclic two-fold symmetry averaging using MAIN.[13] Electron density maps calculated with phases after averaging allowed a detailed interpretation of the binding of compounds **4** and **5**; the complexes were refined to crystallographic free R-factors of 25.6% and 24.5%, respectively. The crystallographic data revealed that both inhibitors form a covalent acyl-enzyme bond with the proteasomal *N*-terminal nucleophilic threonine residue (Figure 2).

Given that both spiro β-lactones **4** and **5** target the same site of the proteasome, the disparity in their potency as inhibitors may be ascribed either to differences in the kinetics of proteasome binding and/or acylation, or to variation in the stabilities of the acyl-enzyme intermediates. Close inspection of the detailed structures of these intermediates provides indirect evidence for the latter possibility.

The acyl-enzyme intermediate derived from potent spiro β-lactone **4** is nearly identical to that of omuralide (Figure 2a). Both structures show β-lactone ring opening by the oxygen of the *N*-terminal threonine residue and exhibit identical stabilizing contacts with the enzyme, including the hydrophobic interaction within the S1 pocket and the key hydrogen bond between the C-6 hydroxyl and the *N*-terminal threonine nitrogen. The similarity of these two acyl-enzyme intermediates does not offer any clues as to the likelihood of interconversion between **4** and **2**; however, it is consistent with the observation that both compounds inhibit the proteasome to approximately the same extent.[14]

Interestingly, the structure of **4** also contains a water molecule cluster (NUC) near the active site that is displaced from its typical location in uninhibited proteasome (Figure 2c).[15] This is the first time the location of the cluster, which plays an important role in the catalysis of peptide-

bond cleavage, has been characterized in the presence of a small-molecule inhibitor. It is also important to note that the C-6 hydroxyl group of **4** lies along the trajectory required for nucleophilic attack on the ester carbonyl group, effectively blocking hydrolysis. Shielding of this key linkage by inhibitor functional groups is also observed in the crystal structures of acyl enzyme intermediates derived from salinosporamide A and the β-lactone based inhibitor homobelactosin C.[16]

Analysis of the structure of acyl-enzyme intermediate arising from the weakly active spiro β-lactone epimer (**5**) provided additional insights (Figure 2b). Surprisingly, this structure is also nearly identical to the omuralide-proteasome complex (Figure 3). With the exception of a much longer C-6 hydroxyl–*N*-terminal threonine hydrogen bond, all of the same stabilizing interactions are present in the structure of **5**. This structure is inconsistent with the hypothesis that the discrepancy in potency between **4** and **5** results from a difference in binding orientation. Rather, it supports the idea that hydrolytic stability may be a key determinant of potency.[17] The major differences between the three structures lie in the area around the C-6 hydroxyl; in the intermediate derived from β-lactone **5**, the opposite stereochemical configuration at C-6 provides an open trajectory for water to attack the C-4 ester bond. Additionally, the increase in the distance between the C-6 hydroxyl and the nucleophilic *N*-terminal threonine nitrogen implies a much weaker hydrogen bond, which would result in enhanced nucleophlicity at nitrogen and facilitate catalysis of hydrolysis.

Comparison to the established mode of action of related β-lactamase and serine protease inhibitors lends support to this interpretation. Penicillic acid derivatives that form stable acyl enzyme intermediates with β-lactamase have been characterized by X-ray crystallography and share many important features with the **4**:20S complex.[18] Most pertinent to the present study, inversion of the stereochemistry of a hydroxyl group adjacent to the ester linkage has been found to dramatically reduce activity in this class of inhibitors.[19] Serine protease inhibitors that act through formation of stable acyl-enzyme intermediates have also been identified. Several serine protease inhibitors are also known to act through formation of stable acyl-enzyme intermediates and prevent ester hydrolysis by engaging active site residues crucial for catalysis in hydrogen bonds. In this series, changing the stereochemistry of the inhibitor functional group involved in the key hydrogen bonding interaction also results in a loss of activity.[20]

The basis for the difference in potency between the epimeric spiro β-lactones may hold implications for the design of new proteasome inhibitors possessing increased activity and selectivity. Interactions with the *N*-terminal threonine nitrogen atom are observed in crystal structures of the entire β-lactone class of proteasome inhibitors, including salinosporamide A and homobelactosin C. Moreover, a similar hydrogen bond to the this key atom is observed in the crystal structure of the proteasome complexed with the peptide boronic acid inhibitor bortezomib.[21] Another class of proteasome inhibitors, the epoxomicins, inactivates the enzyme by forming a covalent bond to this atom.[22] These examples highlight the important role played by the *N*-terminal threonine nitrogen in facilitating proteolysis and suggest that reducing or eliminating the reactivity of this residue is an important strategy for achieving effective proteasome inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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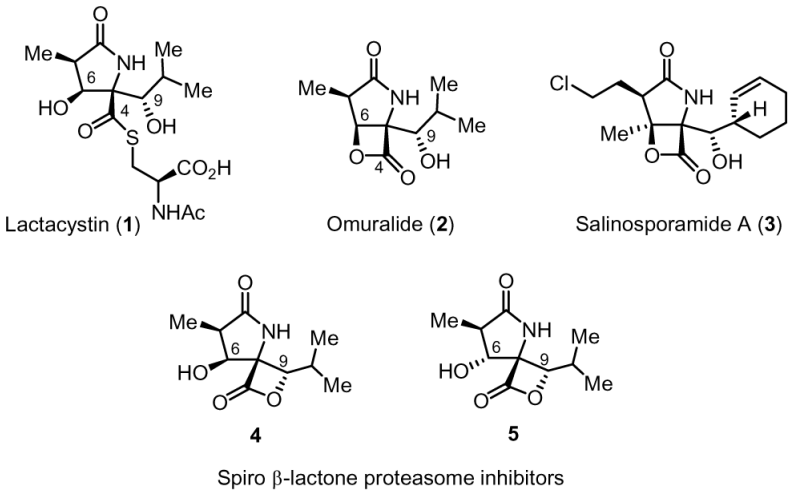

Figure 1.

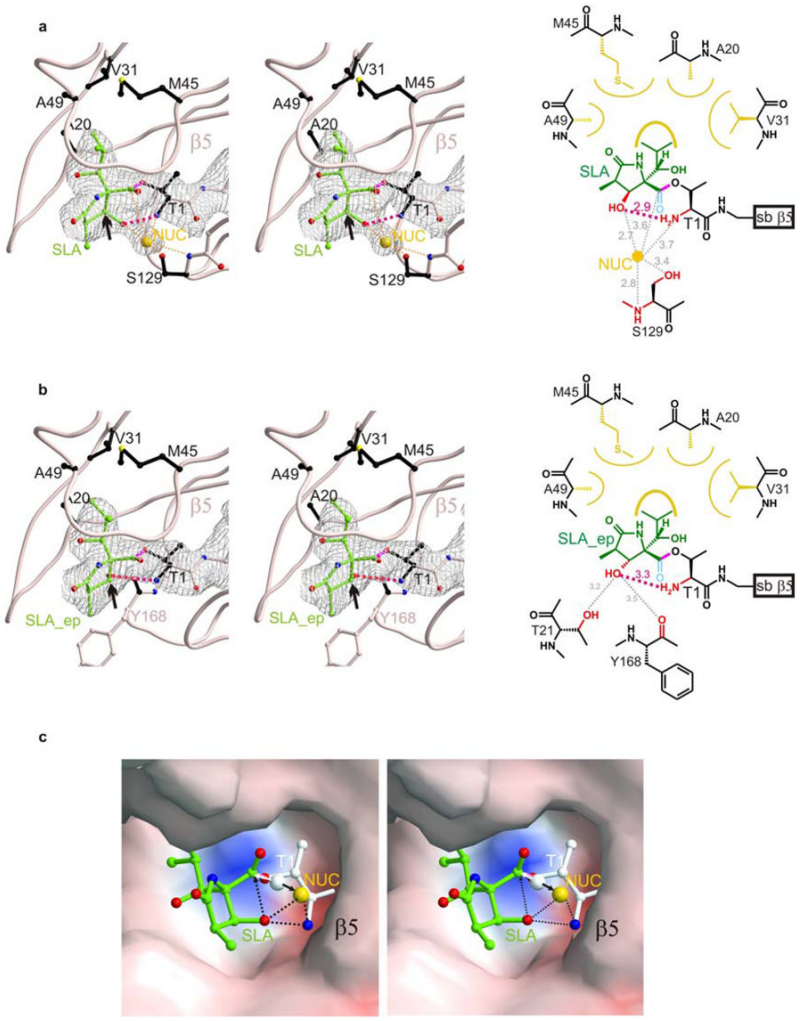

Figure 2.

a) Stereoview of spiro β-lactone **4** bound to the chymotrypsin-like (β5) subunit of the yeast 20S proteasome. b) Stereoview of spiro β-lactone **5** bound to the chymotrypsin-like (β5) subunit of the yeast 20S proteasome. c) Stereoview of spiro β-lactone **4** showing the displacement of bound water cluster (NUC, yellow) from its usual position in the active site of free proteasome (white).

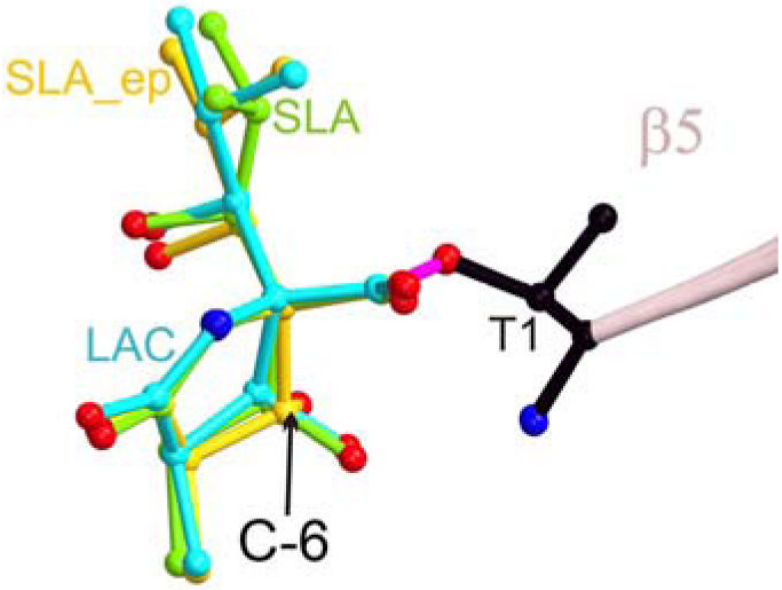

Figure 3.
Overlay of the structures of omuralide (**2**) (LAC), spiro β-lactone **4** (SLA), and spiro β-lactone **10** (SLA_ep) bound to the chymotrypsin-like (β5) subunit of the yeast 20S proteasome.