Solution Structure of Substrate-based Ligands When Bound to Hepatitis C Virus NS3 Protease Domain

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The interactions of the NS3 protease domain with inhibitors that are based on N-terminal cleavage products of peptide substrates were studied by NMR methods. Transferred nuclear Overhauser effect experiments showed that these inhibitors bind the protease in a well defined, extended conformation. Protease-induced line-broadening studies helped identify the segments of inhibitors which come into contact with the protease. A comparison of the NMR data of the free and protease-bound states suggests that these ligands undergo rigidification upon complexation. This work provides the first structure of an inhibitor when bound to NS3 protease and should be valuable for designing more potent inhibitors.

Hepatitis C virus (HCV) infection is an important cause of chronic hepatitis, cirrhosis, hepatocellular carcinoma, and liver failure worldwide (1). Approved therapies with proven benefit for patients with chronic hepatitis C include various drug regimens of interferon-α. These therapies have limited efficacy with a low sustained response rate and frequent side effects (1). Therefore, there is an urgent need for the development of new therapies for the treatment of HCV infections.

HCV is a small enveloped virus containing a single-stranded RNA genome of positive polarity, which encodes a unique polypeptide of approximately 3000 amino acids (for reviews see Refs. 2 and 3). This polypeptide is the precursor of four structural and six nonstructural (NS) proteins (4–10). The structural proteins are proteolytically processed by host signal peptidases, whereas two virally encoded proteases within the NS2 and NS3 regions process the remaining nonstructural proteins.

The NS3 serine protease domain (20 kDa), located within the N-terminal portion of the NS3 protein, mediates the proteolysis at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions (6–11). We and others have recently reported that N-terminal cleavage products of peptide substrates are competitive inhibitors of NS3 protease activity (12, 13), which has served as the basis for designing substrate-based inhibitors (14, 15). To date, there have been no reports in the literature on the structure of substrates or inhibitors when bound to NS3 protease, which would certainly be valuable for inhibitor design efforts. However, x-ray crystal structures have been determined for NS3 protease alone (16) and for NS3 protease in the presence of an NS4A peptide cofactor (17, 18). These structures show that NS3 protease adopts a chymotrypsin/trypsin-like fold.

In this report we applied NMR methods to study the structure of peptides and inhibitors, based on N-terminal cleavage products of peptide substrates when bound to the NS3 protease domain of HCV. Transferred NOESY experiments were used to determine the conformation of ligands when bound to the protease, and differential line-broadening experiments were used to identify which segments of the ligands contact the protease.

EXPERIMENTAL PROCEDURES

Purification of NS3 Protease—A modification of a previously published procedure (19) was used to purify the NS3 protease. E. coli (BL21(DE3) pLyS8, transformed with a pET11d vector expressing amino acids 1–180 of HCV NS3 type 1b, was grown at 37 °C in CircleGrow (BIO 101, Inc., Vista, CA) medium supplemented with 200 μg/ml ampicillin and 34 μg/ml chloramphenicol. At mid-log phase the culture was cooled to 24 °C and induced with 1 mM isopropyl-β-D-thiogalactoside. Three hours post-induction, cells were harvested by centrifugation, and the cell paste was frozen at −80 °C. Following two freeze-thaw cycles the cell paste was resuspended in 3 ml of Buffer A (25 mM NaPO₄, pH 7.5, 5 mM dithiothreitol, 10% glycerol, and 0.1% octyl-β-D-glucoside), 1 mM EDTA, 0.1% octyl-β-D-glucoside, 15 mM NaCl) per gram of cells. The suspension was processed in a Dounce homogenizer, supplemented with 20 mM MgCl₂ and 10 μg/ml Dnase I (bovine pancreatic, Amersham Pharmacia Biotech), and incubated for 20 min on ice. Following a brief sonication, the extract was clarified by a 30-min centrifugation at 14,500 × g. The supernatant was applied to a SP-Sepharose column (equilibrated with 50 mM NaPO₄, pH 6.5, 5 mM dithiothreitol, 10% glycerol, 1 mM EDTA, and 0.1% octyl-β-D-glucoside) and eluted with a 150–1000 mM NaCl gradient. NS3 protease enriched fractions were pooled and diluted with Buffer A (25 mM NaPO₄, pH 7.5, 5 mM dithiothreitol, 10% glycerol, and 0.1% octyl-β-D-glucoside) to decrease the NaCl concentration to 100 mM. The NS3 protease pool was applied to a heparin-Sepharose column and eluted with 0.3 M NaCl in Buffer A. The eluted protein was concentrated and applied to a Superdex 75 column (in Buffer A with 350 mM NaCl) and eluted as a single major peak of homogeneous NS3 protease (Fig. 1). Purified protein preparations were checked by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry, and the enzyme concentration was quantified by PTH-derivatization of a total amino acid hydrolysate of the purified protein. A conversion factor of 1.22 was implemented to correct the protein concentration as determined from a Bio-Rad protein assay using a bovine serum albumin standard. The proportion of enzyme in active form was estimated to be greater than 95% based on a comparison of previously published activities (19, 20).

Inhibitor Synthesis—Peptides 1, 2, and 3 (Table I shows the compounds used in this study) were synthesized using standard solid-phase
methodology (21, 22). Fmoc (N-(9-fluorenylethoxycarbonyl)-protected amino acids and resins were obtained from Nova Biochem, Bachem, or Advanced ChemTech. The synthesis of 4 was carried out in solution using standard peptide chemistry (22). Each compound was purified by preparative reversed-phase HPLC on a C18 column using an acetonitrile gradient. Satisfactory *H NMR, mass spectrometry, amino acid analysis, and homogeneity data (> 90% HPLC) were obtained for all the compounds.

Enzymatic Assay—The enzymatic assay was performed in 50 mM Tris-HCl, pH 7.5, 30% *(v/v) glycerol, 2% *(v/v) CHAPS, 1 mg/ml bovine serum albumin, and 1 mM TCEP. 25 μl of the substrate DDIVPC-SMSY/TW, −1 mM biotin-DDIVPC-SMSY, 125I-labeled TW, and various concentrations of inhibitor were incubated with 11 mM protease for 60 min at 23 °C. These conditions were chosen to obtain < 20% substrate conversion to minimize the effect of product inhibition (12, 13). The final Me2SO concentration did not exceed 6.4%. The reaction was terminated with the addition of 0.025 N NaOH. The separation of substrate from products was performed by adding avidin-coated agarose beads to the assay mixture followed by filtration. A nonlinear curve fit using the Hill equation was performed by adding avidin-coated agarose beads to the assay mixture followed by filtration. A nonlinear curve fit using the Hill equation.

K<sub>i</sub> Determination—The initial velocities were determined at multiple inhibitor and substrate concentrations under the assay conditions described above in the presence of 22 mM protease. To minimize the effect of product inhibition, the cleavage rates were only measured during the initial phase of substrate conversion (<20%). Accordingly, the initial rates were linear and described a Michaelis-Menten kinetics. *K<sub>i</sub> calculations were performed by nonlinear regression analysis of the velocity data using the GraFit software (version 3.0, Erithacus Software Ltd., Staines, UK) and Equation 1 for competitive inhibition.

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V = \frac{V_{\text{max}} S}{(K_{\text{M}} + [S]) + S} \tag{1}
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NMR Methods—Two identical samples were initially prepared for each one-dimensional *H NMR experiment. Sample tubes (5 mm) containing 2 mM inhibitor were prepared by adding 10 μl of concentrated solutions in Me2SO-<sup>d6</sup> to an aqueous buffer composed of 25 mM Na2PO<sub>4</sub>-<sup>d6</sup>, 300 mM NaCl, 5 mM dithiothreitol-d<sub>10</sub>, 10% *(v/v) glycerol-d<sub>10</sub>, and 10% *(v/v) D<sub>2</sub>O spiked with 3-(trimethylsilyl)-propionic 2,2,3,3-d<sub>4</sub>-l. The final pH values of the solutions were adjusted to 7.0, and buffer was added to a final volume of 600 μl. To one of the samples described above was added a concentrated stock solution of NS3 protease (depending on the experiment, the concentration used ranged between 2.1 to 3.6 mg/ml) in a buffer identical to that employed above, with the exception of 0.01% Nonidet P-40 detergent, such that an inhibitor/protease ratio of 30:1 was typically achieved.

All spectra were acquired on a Bruker DRX 600-MHz NMR spectrometer at 30 °C with the exception of the spectra shown in Fig. 2B, which were acquired on a Bruker AMX 400-MHz NMR. Suppression of the solvent signal was achieved by the use of presaturation or by inserting a 3–9-19 WATERGATE module prior to data acquisition (23, 24). Phase-sensitive NOESY, total correlation spectroscopy (TOCSY), and ROESY experiments were acquired using the time-proportional phase incrementation method. NOESY experiments were recorded with mixing times of 100, 200, and 250 ms and a water-selective flip-back pulse prior to the readout pulse (25). The ROESY experiment was recorded with a 250-ms spin-lock period. Two-dimensional data sets were typically acquired with 2048 points in <i>t</i><sub>r</sub> and 400 points in <i>t</i><sub>τ</sub>. 128 scans were averaged for each NOESY <i>t</i><sub>r</sub> increment and 64 scans for each TOCSY <i>t</i><sub>τ</sub> increment. The data were processed and analyzed using XWinNMR and WinNMR software (Bruker Canada, Milton, Ontario) and TRIAD software (Tripos, St. Louis, MO). Data sets were typically zero-filled to yield 2048 × 1024 real points after transformation using a phase-shifted sine bell window function.

One-dimensional 13C spectra for <i>T</i><sub>1</sub> relaxation measurements for 4 (36 mM) were acquired at 150 MHz and 27 °C in Me<sub>2</sub>SO solvent. Inversion recovery experiments were run with power-gated proton decoupling during acquisition. Twelve spectra were acquired corresponding to the <i>r</i> delays (0.01, 0.1, 0.2, 0.5, 0.7, 0.65, 1.0, 1.5, 2.2, 3.0, 5.0, 8.0, and 7.5 s). Each spectrum was acquired by adding 1600 transients and using a relaxation delay of 7.54 s. ROESY and J-coupling data collected in both Me<sub>2</sub>SO solvent and Buffer A, described above, were found to be similar, suggesting that 4 assumes similar conformational properties in both solvents.

Computational Methods—The structure of 4 was modeled by a simulated annealing protocol using Discover 85.0 and the CFF95 force field (Molecular Simulations Inc., San Diego, CA). All calculations were performed without nonbonded or coulombic cut-offs and a dielectric constant of 1.0. NMR-derived distance restraints were generated from the NMR data using a method similar to that of Sykes (26). The 27 restraints derived from NOESEY distance data were applied as strong restraints (1 Å), medium (1.8–3.5 Å), or weak (1.8–5.0 Å) flat-bottomed potentials having force constants of 50 kcal/mol Å<sup>2</sup>. A single, high temperature, unrestrained dynamics run was performed at 1000 K using a time step of 1 fs, with 50 structures collected at 10-ps intervals to generate a starting set of conformations. Each structure was cooled and minimized using the following simulated annealing protocol. The temperature was lowered to 800 K at a rate of 50 K/ps<sup>-1</sup> where strong restraints were applied, followed by additional cooling to 250 K (5 K/ps<sup>-1</sup>). The remaining restraints were added and cooling to 50 K (0.5 K/ps<sup>-1</sup>) was performed followed by restrained minimization to a final gradient of 0.01 kcal/mol Å<sup>-1</sup> Å<sup>-1</sup>. Sixteen low energy, NMR-consistent structures were isolated and are shown superimposed based on the P1–P4 backbone in Fig. 5A. The root mean square deviation for the backbone heavy atoms of P1–P4 (excluding the acid oxygens of P1) is 0.25 Å. The average total restraint violation energy is 0.14 kcal/mol with a S.D. = 0.13 kcal/mol.

RESULTS AND DISCUSSION

Identification of Ligand Segments That Bind to Protease—The differential line-broadening experiment is a powerful NMR method for detecting protein-ligand interactions of moderate affinity (**K<sub>i</sub>** = 10<sup>-3</sup> to 10<sup>-6</sup> M) and for identifying the contact sites on ligands that interact with a target protein (27). The relative ease and simplicity of the method makes it particularly attractive. For a protein-ligand complex in fast exchange on the NMR time scale, one can observe changes in the predominant

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**TABLE 1**

| Name | P10 | P9 | P8 | P7 | P6 | P5 | P4 | P3 | P2 | P1 | P1 | P2 | P2 | P3 | P4 | P4 | P4 | P6 | P6 | IC<sub>50</sub> μM |
|------|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---------|
| 1    | Asp | Asp | Ile | Val | Pro | Cys | Ser | Ser | Ser | Tyr | Thr | Thr | Thr | Thr | Thr | Thr | Thr | Thr | Thr | >2500   |
| 2    | Thr | Glu | Ala | Gly | Asp | Asp | Ile | Val | Pro | Cys | Ser | Met | Ser | Tyr | Thr | Thr | Thr | Thr | Thr | 68     |
| 3    | Thr | Glu | Ala | Gly | Asp | Asp | Ile | Val | Pro | Cys | Ser | Met | Ser | Tyr | Thr | Thr | Thr | Thr | Thr | 34     |
| 4    | Thr | Glu | Ala | Gly | Asp | Asp | Ile | Val | Pro | Cys | Ser | Met | Ser | Tyr | Thr | Thr | Thr | Thr | Thr | 12     |

*Hbp represents 4-trans-benzylxypoline.

* Nva represents norvaline.
FIG. 2. Differential line-broadening experiments for studying interactions of inhibitors with NS3 protease. Shown are selected subregions of $^1$H spectra (scaled as ppm) of inhibitors in the absence (blue) and in the presence (red) of NS3 protease (at an inhibitor to protease...
ligand resonances upon addition of less than stoichiometric amounts of protein (e.g. 30:1 ligand to protein ratio). The observed protein-induced changes in ligand resonances are due to the different environments of the ligand hydrogen nuclei in the free and the bound states.

We have previously described differential line-broadening and transferred NOESY NMR methods that were successful for determining the structures of inhibitors (28) and peptides (29) (based on N-terminal cleavage products of peptide substrates) when bound to human cytomegalovirus serine protease. Based on this experience, our initial aim was to identify whether the N-terminal cleavage products of peptide substrates of NS3 protease also exhibited fast exchange appropriate for line-broadening experiments. The N-terminal cleavage product of an NS5A/5B-derived peptide substrate (1, Table I) was tested for inhibitory activity. The moderate affinity of 1 (IC50 = 68 μM) for NS3 protease suggested that this competitive inhibitor (12) would be a good candidate for line-broadening and transferred NOESY studies.

A differential line-broadening study began by acquiring 1H NMR spectra of 1 (Fig. 2A) in the absence (blue) and presence of the protease (red). A comparison of these spectra shows changes to specific resonances of 1 (Fig. 2A) that results from fast-exchange binding to the protease (on the NMR time scale). The specific nature of the protease-induced perturbations shown in Fig. 2A suggests that these segments of 1 likely contact the enzyme (e.g. see the resonances of P4 αH, P1 βCH2, and P4 γCH3/δCH3; residue positions are labeled according to the nomenclature described in Ref. 30). Other resonances of nonexchangeable hydrogens did not exhibit significant perturbations upon addition of the protease (e.g. P6 αH and P3 γCH3 in Fig. 2A). These hydrogen atoms do not experience significantly different environments in their free and bound states and may not directly contact the enzyme.

In contrast to the observation of N-terminal product inhibition, peptide 2, which is based on the C-terminal cleavage product of the NS5A/5B-derived peptide substrate, does not bind to the protease. Fig. 2B shows no difference between the spectrum of free 2 (blue) and the spectrum of 2 in the presence of protease (red). Moreover, peptide 2 does not inhibit NS3 protease activity (IC50 > 2500 μM).

To investigate other ligand contact points with NS3 protease, a line-broadening study was performed on 3 (IC50 = 34 μM). This compound is based largely on the P10–P1 sequence of the NSSA/5B cleavage site. A comparison of transferred NOESY data (not shown) and line-broadening patterns for 1 and 3 in the presence of the protease shows that the P1–P6 residues bind the protease in a similar mode (e.g. 1 and 3 have similar broadening patterns for P1 βCH2, P3 γCH3, and P4 γCH3/δCH3, shown in Fig. 2, A and C). However, the lack of protease-induced resonance perturbations for the residues comprising P7–P10 indicate the lack of significant contacts between these residues and the protease (e.g. see the resonances labeled P8 βCH3 and P10 δCH3 in Fig. 2C). These results are consistent with the observation that extension of the hexapeptide 1 N-terminus does not increase inhibitory potency (12).

Further along in our efforts to design potent inhibitors, we found that a (4R)-benzoyloxy substituent on the P2 proline and in which the P1 cysteine was replaced by the chemically more stable norvaline (14). Although the latter replacement typically reduces the potency of inhibitors (14), it does favor fast-exchange binding, which is required for line-broadening and transferred NOESY experiments. A rapid, reversible binding of 4 to the protease was then confirmed by comparing the 1H spectra in Fig. 2D of free 4 (blue) to that of 4 in the presence of protease (red). Specific binding to the active site of the protease was also demonstrated by kinetics and NMR studies. The Dixon and the Cornish-Bowden plots (31) obtained with 4 (Fig. 3) are characteristic of a competitive mode of inhibition. Furthermore, the best fit observed by nonlinear regression analysis of these data also shows that 4 competitively inhibits the NS3 protease with a Ki of 5.4 μM. Also, NMR data illustrate that the protease-induced line broadening observed in Fig. 2D is lost (black spectrum in Fig. 2E) upon addition of a more potent inhibitor (IC50 = 0.041 μM).

The fortuitous resonance dispersion in the 1H spectrum of 4 allowed us to identify most of the hydrogen resonances that are perturbed by the protease. For example, Fig. 2D shows that the P2 aromatic ring resonances of free 4 were significantly altered with the addition of the protease, suggesting that this group contacts the protease. Many of the resonances of the side chains of P1, P2, and P4 also exhibited protease-induced line broadening, which helps explain their role in the binding of 4 to the protease. A summary of the line-broadening data for resonances of P1 to P4 is illustrated in Fig. 5C and discussed later. Interestingly, little or no broadening was observed for the side chains of P3 (e.g. see Fig. 2D), P5, and P6. The lack of protease-induced broadening for the P3 side chain resonances suggests a minimal role in direct binding by this group. It also highlights the confomational role that P3 likely plays in orienting the main chain in the free state for optimal binding to the protease (see below). The exact role of P5 and P6 in binding to the

![Image](https://via.placeholder.com/150)
protease is still unclear. The resonances of these residues lack significant protease-induced broadening for the nonexchangeable hydrogen atoms, yet these residues are important for optimal inhibitor potency (12). This would suggest that the acids of the side chains may have an important binding role. Unfortunately, this can not be tested using these NMR methods because of the lack of observable $^1$H resonances for rapidly exchanging hydrogen atoms. However, we have shown elsewhere that the aspartic acid at P5 can be replaced by tert-butyglycine with no loss in inhibitory potency (12). On the other hand, replacement of the aspartic acid at P6 by alanine results in a significant loss in inhibitory potency. The importance of this acid group is highlighted by the fact that sequence comparisons among isolated HCV strains have revealed a structurally conserved acid residue at the P6 position (10).

Solution Structure of 4 When Bound to Protease—We applied transferred NOESY methods to determine the structure of 4 when bound to NS3 protease. As a control experiment, a NOESY spectrum of free 4 was acquired. Fig. 4B shows a subregion that contains only a few distance-related cross-peaks, as expected for a small molecule of this size. A subsequent NOESY spectrum (Fig. 4A) of the same sample, following addition of NS3 protease (30:1 inhibitor to protease ratio), exhibited many new negative cross-peaks, as expected for a small molecule when bound to a large, slow tumbling macromolecule. Also, the negative cross-peaks present in the spectrum of free 4 (Fig. 4B) were more intense in the transferred NOESY spectrum (Fig. 4A). These results indicate a well-defined and rapidly reversible binding of 4 to the protease. Other changes included the loss of artifact zero quantum cross-peaks (e.g. P1-NH/P1-αH, P3-NH/P3-αH, and P4-NH/P4-αH) observed for free 4 (Fig. 4B) and their replacement with negative NOESY cross-peaks (Fig. 4A) in the presence of the protease. Upon addition of a more potent inhibitor (IC$_{50} = 0.041$ μM) that blocks the active site, the transferred NOESY data of 4 in the bound state is lost (Fig. 4C), and the resulting spectrum resembles that of free 4 (compare Fig. 4, C and B).

Having demonstrated that the NOESY cross-peaks in Fig. 4A arise from 4 when bound to the protease, an NMR-derived structure of enzyme-bound 4 was obtained through restrained simulated annealing techniques. The NOESY cross-peak volumes were scaled and converted to interproton distance restraints (similar to a method described in Ref. 26). Fig. 5A shows 16 low energy, transferred NOESY-derived structures that are superimposed from P1 to P4. An extended peptide conformation is clearly evident from P1 to P4 in all of the structures with only minor deviations along the backbone chain. The root mean square deviation for the backbone heavy atoms of P1–P4 (excluding the acid oxygens of P1) is 0.25 Å. Small deviations in the side chain conformations of P1, P3, and P4 also suggest well defined structures for these residues. The P2 proline ring is also well defined, and the benzyloxy group is observed only in the pseudo-axial conformation. The large variation of positions for the P2 benzyloxy substituent is a result of the lack of sufficient restraints between the benzyloxy group and the rest of the inhibitor. The large variation of positions of P5 and P6 is also due to the lack of sufficient restraints. However, a strong NOESY cross-peak exists between the P4 NH and the P5 αH (Fig. 4A), and an inter-residue side chain NOESY cross-peak is found between P5 βH and P3 γCH$_3$. The observation of only a few transferred NOESY cross-peaks involving P5 and P6 suggests that they may not be as well structured in the bound state. This may also explain why the P5 and P6 residues experience little or no protease-induced line-broadening. Finally, comparisons of all the NMR data acquired for 4 with those of 1 and 3 show striking similarities which strongly suggests that these inhibitors bind to the NS3 protease with the same binding mode.

Protease-binding Face of 4—With a representative structure (Fig. 5B), it is possible to propose the protease-binding face of 4 after considering all of the differential line-broadening data. Fig. 5C displays the hydrogen atoms of the representative...
structure colored in red when significant line broadening was observed, and colored in blue when little or no line broadening was observed. A gray-colored hydrogen atom indicated that resonance overlap hindered an unambiguous distinction. The side view of 4 (Fig. 5D) clearly shows, with few exceptions, that the lower half of the structure has a predominance of hydrogen atoms for which line-broadening effects were observed (red), suggesting that this is the face of the inhibitor that contacts the protease. It is also evident that the upper half of the structure has a predominance of hydrogen atoms for which no significant line broadening was observed (blue), suggesting that, in the bound state, this face may be pointing toward solvent (Fig. 5D).

It may be possible to apply this structural information for designing novel, nonpeptidic inhibitors by replacing parts of the peptidic backbone with a new scaffold that connects the groups that are critical for binding to the protease.

The conformation and proposed binding face of 4 is consistent with the commonly known binding mode of substrates/inhibitors to serine proteases (for a review see Ref. 32). Serine proteases bind their substrates/inhibitors in an extended conformation (forming an antiparallel sheet) through a set of critical hydrogen bonds between a β-strand that lies at the bottom of the substrate binding pocket and the P3 backbone amide and carbonyl. In our structure, the P3 backbone amide and carbonyl of 4 are pointed toward the protease binding face.

Differences in the Conformation of 4 When in the Free versus the Protease-bound State—Another strength of NMR spectroscopy is the potential for identifying differences in structural features of a ligand between the free and bound states. This type of information could be valuable for designing more potent inhibitors by chemically restricting the free conformation to resemble the bound conformation.

Thus far, it has been shown that 4 adopts a well-defined extended conformation in the bound state. In the free state, 4 experiences conformational flexibility such that the backbone adopts a fluid but predominant extended conformation. Evidence comes in part from the intense, inter-residue, backbone cross-peaks in the free state (Fig. 4D, P1-NH/P2-OH, P3-NH/P4-OH, and P4-NH/P5-OH) as compared with similar but stronger cross-peaks in the bound state (Fig. 4A). Consistent with these data are the relatively large coupling constants given in Table II for 4 in the free state. Also, 13C T1 relaxation data were measured to further evaluate the conformational flexibility of 4 in the free state. 13C T1 relaxation is sensitive to motions on the pico- to nanosecond time scale (33, 34). The NT1 values (the product of the number of attached protons and the longitudinal relaxation time T1) in Fig. 6 shows that the main chain-protonated carbons have slower segmental motion (lower NT1 values) compared with the more flexible side chain carbons.

The similarity in backbone conformations in the free and bound states likely facilitates the antiparallel β-sheet interaction, which is typical of substrate binding to serine proteases. A bulky side chain at P3 certainly plays a major role in rigidifying and predefining an extended conformation in the free state (35). This rigidification minimizes the conformational changes of the ligand that may be required for making the critical hydrogen bonds between the protease and the P3 amide and carbonyl in the complex. This is consistent with the observation that the P3 position is highly sensitive in terms of a decrease in inhibitor potency when the P1–P6 side chains are systematically replaced by alanine residues (12). The valine replacement by alanine at P3 likely introduces a higher degree of flexibility in the backbone of the inhibitor.

The side chains of 4 experience significant segmental motion in the free state and likely undergo immobilization upon binding to the protease. The 13C T1 relaxation data given in Fig. 6 clearly shows that the side chain protonated carbons have faster segmental motion (higher NT1 values) than those of the main chain. For example, the NT1 values of P1 increase from the α to δ carbons. Comparisons of the NOESY spectrum of the bound state (Fig. 4A) with the ROESY spectrum of the free state (Fig. 4D) also show evidence of immobilization of the side chains of P1, P3, and P4 upon binding the protease. Many cross-peaks either appear or become more intense in the NOESY spectrum. However, the data involving the P5 and P6 residues are similar in both the free and bound states (e.g., note the similar intensities of the P5-NH/P6-OH cross-peaks in Fig.
4, A and D), which is consistent with these residues having poorly defined structure in the bound state.

This study provides the first reported structure of an inhibitor when bound to NS3 protease domain, which should be useful for the design of more potent inhibitors. Furthermore, a practical strategy is demonstrated in which NMR methods (line-broadening and transferred NOESY) were used to probe inhibitor binding characteristics in a timely fashion to support medicinal chemistry efforts.

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Fig. 6. 13C NMR relaxation data are presented for 4. NT, data (the product of the number of attached protons and the longitudinal relaxation time) are shown next to the respective compound 4 carbon atoms. A asterisk next to an NT value indicates that resonance overlap was observed for that carbon atom in the 13C NMR spectrum.

Table II

| Residue | Chemical shifta | Others | Scalar coupling values |
|---------|-----------------|--------|----------------------|
| P1      | 8.02            | 1.65  | 1.73 ppm             |
| P2      | 4.50            | HJb 2.49 |
|         | 2.13 ppm        |
| P3      | 8.24            | 4.45  | 2.05                 |
| P4      | 7.94            | 4.12  | 1.84                 |
| P5      | 8.44            | 4.59  | 2.66, 2.59           |
| P6      | 8.25            | 4.59  | 2.69, 2.54           |
| Ac      |                 |        | 2.05                 |

a 1H chemical shifts were referenced to an internal standard: 3-(trimethylsilyl)-propionic-2,2,3,3-d4 ac acid sodium salt at 0.00 ppm at 30 °C.

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