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NMR Line-Broadening and Transferred NOESY as a Medicinal Chemistry Tool for Studying Inhibitors of the Hepatitis C Virus NS3 Protease Domain

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Abstract—This work describes the use of NMR as a medicinal chemistry tool for better understanding the binding characteristics of inhibitors of the HCV NS3 protease. The protease-bound structure of a tetrapeptide-like inhibitor that has an acid C-terminus, a norvaline at P1 and a naphthylmethoxy proline at P2 is described. Conformational comparisons are made with a similar compound having a 1-amino-cyclopropylcarboxylic acid at P1 and with a hexapeptide inhibitor. Differences between the free and bound states are identified. ^{19}F NMR also helped in determining that a single complex is observed when an inhibitor is added to the protease at a 1:1 ratio.

Hepatitis C virus (HCV) infection is an important cause of chronic hepatitis, cirrhosis, hepatocellular carcinoma and liver failure worldwide.[1] It has been estimated that the virus infects approximately 3% of the human population.[1] There is, therefore, an urgent need for the development of new and efficacious 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 polyprotein of approximately 3000 amino acids.[2] Two virally-encoded proteases within the non-structural (NS) region, NS2 and NS3, mediate cleavages to produce the non-structural mature proteins. The NS3 serine protease domain (20 kDa) is located within the amino-terminal portion of the NS3 protein, and its activity is enhanced by the NS4A protein. A similar enhancement of activity is observed when synthetic peptides, encompassing residues 21 to 34 of the NS4A protein, are added to the enzyme.[3] The solution structure of the free protease domain has been determined using NMR spectroscopy, and it was proposed that the cofactor activates the enzyme by stabilizing the fold of its N-terminal domain.[4]

The NS3 protease is required for viral replication (as demonstrated in the chimpanzee),[5] and inhibition of this protease has been considered as a suitable strategy for the development of antiviral pharmaceuticals.[6] We and others have recently reported that N-terminal cleavage products of peptide substrates are competitive inhibitors of NS3 protease activity, which has served as the basis for designing substrate-based inhibitors.[7]

Our inhibitor design strategy focused on improving the potency and on decreasing the peptidic character of these hexapeptides. The preceding Letter shows that an important improvement in potency was attained with the addition of a (4*R*)-1-benzyloxy substituent on the P2 proline. An NMR line-broadening and transferred NOESY study[8] suggested that the benzyl group binds to the protease at a distinct site. The ^{1}H resonances of the benzyl group were significantly line-broadened upon addition of the protease to the sample tube, and there were no intra-ligand transferred NOESY peaks observed with the other residues. New inhibitors were

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subsequently designed to further probe this new binding site. The preceding Letter shows that replacement of the benzyl for a larger naphthyl group results in inhibitors having improved potency.[9] The same NMR study was also helpful in designing less peptidic inhibitors. Line-broadening and transferred NOESY data suggested that the most critical residues for binding are P1 to P4, and the preceding letter demonstrates that tetrapeptide-like inhibitors with reasonable potency can be made.[9]

This work shows that compound **1** (Fig. 1) binds the NS3 protease in an extended conformation. Potential contact sites with the protease active-site are also described. Similar binding features are observed for compound **2**. Based on transferred NOESY data, the bound structures of compound **1** and **2** indicate that the naphthyl group and the P4 side chain lie close to one another. A comparison of the bound structure with the free state indicates that significant differences exist. ^{19}F NMR experiments were also performed to ensure that an inhibitor binds to the protease as a single complex.

Structural Features of Compound 1

Compound **1** is a truncated, tetrapeptide-like inhibitor that has an acid C-terminus, a norvaline at P1, a (4*R*)-1-naphthylmethoxy proline at P2, and a carboxypropanoyl N-terminus. Transferred NOESY data were first collected to determine the distances between the hydrogens of compound **1** when bound to the NS3 protease domain.[10] Table 1 gives a subset of these distances. NMR-derived structures were then calculated using the collection of distances along with molecular modeling techniques.[10]

Figure 2A displays three representative, low-energy, transferred NOESY-derived structures of compound **1** when bound to the protease. A well-defined, extended

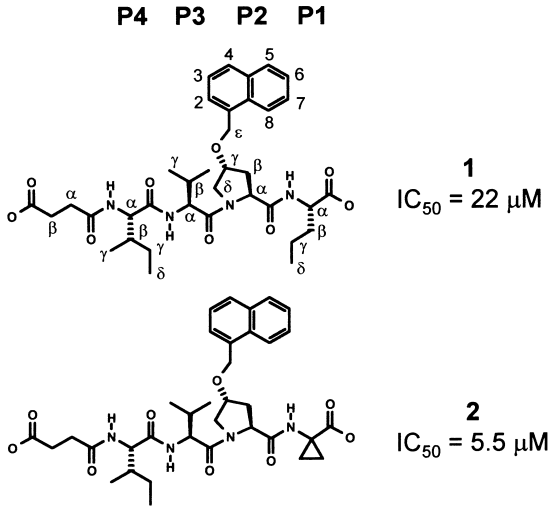

Figure 1. The primary structures of two inhibitors of NS3 protease are given. The nomenclature used in the text is given beside compound **1**. IC_{50} values against the NS3-4A_{pep} protease were determined using an assay described elsewhere.[7]

Table 1. Distances derived from transferred NOESY data of compounds **1** and **2** when bound to the NS3 protease domain[a]

Crosspeak	Apparent distances (Å)	
	Compound **1**	Compound **2**
P1NH-P2αH	2.2	2.1
P2αH-P2βH′	2.3	2.4
P2H2-P4γCH_3	3.2	3.2
P2H3-P4γCH_3	3.5	3.5
P2δH-P2δH′	2.0	1.8
P2δH-P2βH	2.7	2.7
P2εH/H′-P2βH′	2.7	2.7
P2γH-P2βH	2.4	2.3
P2γH-P2βH′	3.0	2.6
P2γH-P2δH	2.5	2.6
P3αH-P2δH	2.5	2.7
P3αH-P3γCH_3	2.6	2.8
P3αH-P3βH	2.6	2.8
P3NH-P3βH	2.5	2.7
P3NH-P3γCH_3	3.0	3.3
P3NH-P4αH	2.1	2.1
P4NH-P5αH	2.7	2.9

[a]The distances listed here are a subset of the full list identified for both molecules. The subset was limited to those crosspeaks which were common to both compounds **1** and **2** and which were unobstructed by peak overlap or noise. A series of transferred NOESY data were collected at 800 MHz and 303 K.

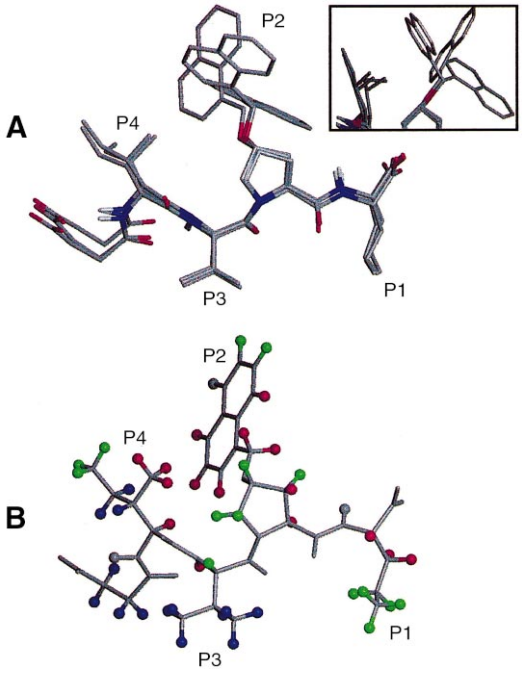

Figure 2. NMR-derived structure of compound **1** when bound to the NS3 protease of genotype 1b. (A) shown are three representative structures determined by restrained simulated annealing. The structures are colored by atom-type (oxygen is red, nitrogen is blue, carbon is dark gray, and hydrogen is light gray). Only the hydrogens of the amides are shown. A side-view of a portion of P2 is given within the box. (B) a qualitative summary of line-broadening observations is illustrated on a representative structure based on comparisons between ^1H spectra of **1** in the absence and presence of the NS3 protease. Relatively no line broadening was observed for hydrogens colored blue, significant line broadening was observed for hydrogens colored red, intermediate line-broadening was observed for hydrogens colored green, and hydrogens were colored gray when resonance overlap hindered a definitive interpretation.

peptide conformation is clearly evident, which is similar to that observed previously for hexapeptide analogues.[8] The naphthylmethoxy substituent in the P2 proline also assumes a pseudo-axial conformation. Although the precise orientation of the naphthylmethoxy group could not be determined, a restricted range of conformations is illustrated in the frame given beside the structures in Figure 2A.

Interestingly, new transferred NOESY crosspeaks were observed between the P4γCH_3 and the P2 naphthyl H3 and H2 resonances, indicating that these groups lie close to one another in the bound state. Similar crosspeaks were not observed for a hexapeptide inhibitor that has a smaller, benzyl substituent at P2.[8] Others have reported inhibitors of the NS3 protease that exploit this proximity by linking the P2 and P4 regions in a macrocycle.[11]

Another intriguing transferred NOESY crosspeak was observed between P1αH and P1δCH_3 indicating that the norvaline side chain folds back or 'curls up' upon binding to the protease. A similar crosspeak was identified for the hexapeptide inhibitors.[8] These observations are consistent with our medicinal chemistry studies. Compounds that have a cyclopropyl P1 side chain exhibit improved potency as compared to inhibitors that have a linear P1 side chain (see Fig. 1 and ref 9). Also, cyclopentyl and cyclobutyl amino acid derivatives at P1 are well-tolerated (unpublished data).

Protease-induced, resonance line-broadening also provided critical information on the binding properties of compound **1**. These results are qualitatively summarized on a representative structure shown in Figure 2B. The hydrogen resonances that are perturbed most by the presence of the protease, and thus are likely at or near the binding interface, are colored red (i.e., αH and βH of P1, the αH and βH of P2, the naphthyl of P2, the NH of P3, and the αH and the γCH_3 of P4). Similar line-broadening patterns were observed for hexapeptide inhibitors and for substrate peptides.[8] Fortunately, the resonances of the naphthyl group are well resolved and exhibit a distinct line-broadening pattern. Strong line-broadening was observed for the P2 naphthyl resonances of H3 and H2, and coupled with the observation of transferred NOESY crosspeaks with P4, suggests that this part of the ring is pointing toward the protease binding face. No line-broadening was observed for the resonances of the N-terminal aliphatic linker, and our Structure–activity relationship (SAR) shows that it can be removed with minimal loss in potency as shown in the preceding letter.[9]

The identification of conformational differences between the free and bound states of an inhibitor should also be useful for designing inhibitors with improved affinity. Comparisons of distance information between the free state (from a ROESY spectrum) and the bound state (from transferred NOESY data) showed significant differences for compound **1**. Conformational changes were observed for the side chain of P1, the P1 NH to P2 βH structure, the linker between the naphthyl and prolyl, and the χ_1 torsion angle of P4. This data is also consistent with the findings of a new NMR technique, transferred ^{13}C spin-lattice relaxation, which identified sites of a hexapeptide inhibitor that rigidified upon binding to the protease (unpublished data). The design of compounds that take advantage of this observation will be described elsewhere.

Structural Features of Compound 2

The preceding Letter demonstrates that an improvement in inhibitor potency is observed when a norvaline at P1 is replaced by a 1-amino-cyclopropylcarboxylic acid. This prompted us to compare the protease-bound conformations of compounds **1** and **2**, which differ chemically at P1 (Fig. 1). A subset of distances derived from the protease-bound state is given in Table 1. A comparison of the distances for compounds **1** and **2** indicates that both bind to the protease in similar conformations, suggesting that the source for the difference in potency between these compounds is likely localized at P1. Unfortunately, the cyclopropyl resonances of compound **2** could not be assigned to distinct hydrogens, which prevented a determination of the precise bound conformation of this group.

Interesting conformational differences were also found when comparing the free and bound states of compound **2**. In the ROESY spectrum (data not shown) of the free state, many crosspeaks are observed between the P1 NH with the P1 cyclopropyl and P2 βH resonances. In the transferred NOESY spectrum of the bound state, these crosspeaks are either lost or change in intensity as would be expected of a more fixed conformation. Chemically rigidifying the P1 to the bound conformation may improve the affinity for the protease.

NS4A Peptide Cofactor

We also applied NMR methods to determine whether the protease-bound conformation of our ligands would differ significantly in a ternary complex involving an NS4A peptide cofactor. Transferred NOESY spectra were collected for compounds **1** and **2** using the NS3 protease domain in the absence and presence of the peptide cofactor. A comparison of the distances calculated from the spectra of bound inhibitors showed mostly minor differences that involved the P2 and P4 residues (data not shown). The distances were consistent with an extended conformation.

^{19}F NMR to Monitor a 1:1 Complex

The transferred NOESY experiments described above employed an inhibitor to protease ratio of 75:1, which is typical for this type of experiment.[12] The NMR signals of the free inhibitor were indirectly used to monitor the information from the bound state. If multiple bound conformations existed, then the transferred NOESY data would report an average of these conformations. We applied ^{19}F NMR to directly monitor the binding

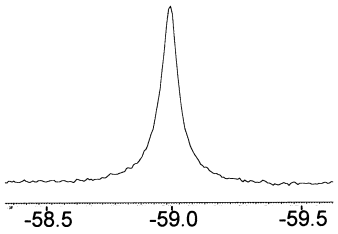

Figure 3. ^{19}F NMR spectra (376.5 MHz and 300 K) of a ^{19}F-labeled inhibitor when bound to the NS3 protease. The protease used for the fluorine study was derived from the BK construct which encoded a modified BK strain sequence that spanned amino acids 1–180 and contained a C-terminal solubilization motif (ASKKKK) as described elsewhere.[7B] The sample used in this study contained 0.7 mM protease in a buffer composed of 100 mM sodium phosphate pH 6.5, 300 mM sodium chloride, 1 mM DTT-d_{10}, and 6% D_2O. Trifluoromethyltoluene was used as an external reference at −63 ppm. The ^{19}F chemical shift of the free inhibitor was found at 59.8 ppm.

stoichiometry of an inhibitor when bound to the protease (i.e., at a 1:1 ratio). Fluorine spectroscopy offers the advantages that fluorine nuclei are very sensitive to differences in the surrounding environment, and resonances will be observed only for those sites that are intentionally labeled with fluorine atoms. Figure 3 shows a fluorine NMR spectrum of the protease in the presence of a ^{19}F-labeled inhibitor, which is similar to compound **2** and contains a single trifluoromethyl group on the P2 aromatic substituent. The observation of only one protease-bound peak at −59.0 ppm is consistent with the presence of a single complex. Furthermore, this series of compounds are competitive inhibitors of the enzyme as described in the previous Letter.[9]

Conclusion

This work describes the application of NMR as a tool for medicinal chemistry in studying inhibitors of NS3 protease. Transferred NOESY and line-broadening data were used to determine the protease-bound conformation of inhibitors and the contact sites with the protease. Comparisons between the ROESY and the transferred NOESY data helped identify conformational differences between the free and bound states. ^{19}F NMR was employed to directly monitor the binding of an inhibitor to the protease.

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