Structure−Activity Study on a Novel Series of Macrocyclic Inhibitors of the Hepatitis C Virus NS3 Protease Leading to the Discovery of BILN 2061

Montse Llinàs-Brunet,*,† Murray D. Bailey,† Gordon Bolger,‡ Christian Brochu,† Anne-Marie Faucher,† Jean Marie Ferland,† Michel Garneau,‡ Elise Ghiro,† Vida Gorys,† Chantal Grand-Maître,† Ted Halmos,† Nicole Lapeyre-Paquette,‡ Francine Liard,‡ Martin Poirier,† Manon Rhéaume,‡ Youla S. Tsantrizos,† and Daniel Lamarre‡

Departments of Chemistry and Biological Sciences, Boehringer Ingelheim (Canada) Ltd., 2100 Cunard Street, Laval, Québec H7S 2G5, Canada

Received November 20, 2003

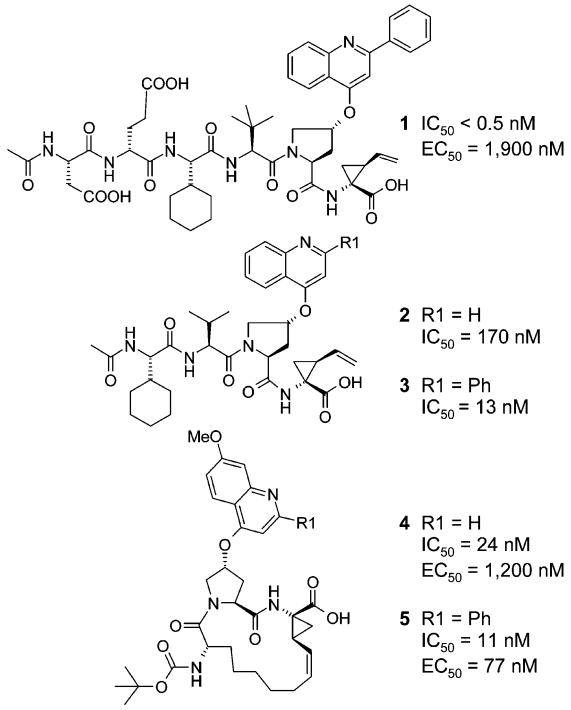
Abstract: From the discovery of competitive hexapeptide inhibitors, potent and selective HCV NS3 protease macrocyclic inhibitors have been identified. Structure−activity relationship studies were performed focusing on optimizing the N-terminal carbamate and the aromatic substituent on the (4*R*)-hydroxyproline moiety. Inhibitors meeting the potency criteria in the cell-based assay and with improved oral bioavailability in rats were identified. BILN 2061 was selected as the best compound, the first NS3 protease inhibitor reported with antiviral activity in man.

Introduction. Hepatitis C virus (HCV) is the leading cause of chronic liver disease worldwide.[1,2] It is estimated that 3% of the world population is infected with this virus.[3] Currently, the most effective therapy consists of using PEGylated α-interferon in combination with ribavirin, giving a sustained virological response of ∼50% in genotype 1 infected patients.[4] The existing therapies are associated with considerable side effects and lead to discontinuation of treatment in certain patient populations. The limited efficacy and side effects of current therapies, linked to the high prevalence of infection worldwide, clearly highlight the need for new therapeutics.

HCV is a small enveloped positive stranded RNA virus that encodes a polyprotein of ∼3000 amino acids. The polyprotein consists of four structural and six nonstructural (NS) proteins.[5] The structural proteins are processed by host peptidases, whereas the NS proteins are processed by two virally encoded proteases, the NS2/3 and NS3 proteases. The NS2/3 protease is responsible for the cleavage between NS2 and NS3, whereas the NS3 protease is responsible for the release of the remaining nonstructural proteins.[6] The NS3 protease, located at the N-terminal 180 amino acids of the NS3 protein, is a chymotrypsin/trypsin-like serine protease.[7] The function and structure of the NS3 protease have been studied in great detail, making this enzyme perhaps the best understood HCV enzyme.[8] The essentiality of this enzyme for viral replication has been demonstrated by the nonproductive infection following liver inoculation of chimpanzees with a genomic HCV RNA mutated in the NS3 protease active site.[9]

Early structure−activity studies were fueled by the discovery of N-terminal product inhibition of NS3 protease by us[10] and others.[11] The interaction between the NS3 protease and the C-terminal carboxylic acid hexapeptide inhibitor is characterized by a series of weak contacts that extend from the P1 to the P6 position. A cysteine is required at the P1 position, and the interaction relies on high charge density especially at the P6 position. Early structure−activity studies led to the identification of potent hexapeptides[12,13] (**1**) followed by tetrapeptides[14,15] (**2**, **3**). More recently, macrocyclic tripeptide inhibitors were identified, as exemplified by **4** and **5**[16] (Table 1).

Table 1. Peptidomimetic Inhibitors of the NS3 Protease

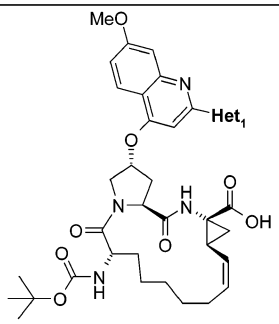


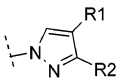
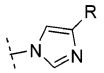
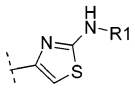
Macrocyclic inhibitor **5** is a selective inhibitor of HCV RNA replication in the cell-based assay (HCV replicon) with an EC_{50} of 77 nM. Compound **5** suffered from an unfavorable pharmacokinetic profile when studied in rats. It is cleared very rapidly from rat plasma (half-life $T_{1/2} = 0.4$ h) and is poorly bioavailable ($F = 2\%$), as reflected by the low plasma concentration (area under the plasma concentration−time curve, $AUC_{0-\infty} = 0.2$ μM·h) following a single oral dose of 25 mg/kg in rats.[16] The main goal of this study was to identify selective NS3 protease inhibitors with low nanomolar cell-based potency (target $EC_{50} < 10$ nM) and with an adequate pharmacokinetic profile for the selection of an orally bioavailable HCV protease inhibitor for further development. This communication describes the optimization of **5** leading to the discovery of BILN 2061.[17]

* To whom correspondence should be addressed. Phone: 450-682-4640. Fax: 450-682-4189. E-mail: mllinas@lav.boehringer-ingelheim.com.
† Department of Chemistry.
‡ Department of Biological Sciences.

10.1021/jm0342414 CCC: $27.50
Published on Web 03/02/2004

Table 2. Macrocyclic Inhibitors with Different Five-Membered Ring Heterocycles at the C2 Position of the Quinoline



Het_1	Compd.		IC_{50}(nM)	EC_{50}(nM)
	**6**		12	200
	**7**		5.1	9.5
	**8**	R1 = R2 = H	5.5	3.9
	9	R1 = H, R2 = Me	3.0	4.0
	10	R1 = Me, R2 = H	22	20
	**11**	R = H	4.7	13
	12	R = Me	14	39
	**13**	R1 = H	5.5	3.2
	14	R1 = Me	6.4	6.0
	15	R1 = -COMe	4.8	4.5

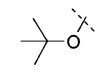
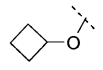
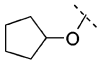
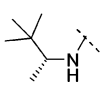
Results. Studies on the tripeptide macrocyclic inhibitor series demonstrated that the 15-membered heterocycle is one of the optimal ring sizes.[18] The C-terminal carboxylic acid is maintained because it contributes considerably to the potency and specificity of the inhibitors.[19] Therefore, SAR studies focused on two pharmacophores: the novel tricyclic substituent on the (4*R*)-hydroxyproline moiety and the left-hand-side carbamate group (capping group).

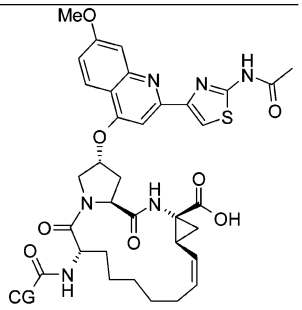
The aromatic substituent in our previously reported acyclic inhibitors has been extensively studied.[12,20−22] Combined efforts between medicinal chemistry and structural research led to the identification of 2-phenyl-7-methoxy-4-quinolinoxy as the optimal group.[14] In particular, when combined with the optimal P1 residue (1*R*,2*S*)-1-amino-2-vinylcyclopropylcarboxylic acid,[15] this substituent produced very potent inhibitors of the NS3 protease. In the macrocyclic inhibitor series, the addition of a C2-phenyl ring on the quinoline produced inhibitors with a modest increase in enzymatic potency (2- to 3-fold) but with greater than 15-fold difference in the cell-based potency (EC_{50} of inhibitors **4** and **5**). The introduction of the 2-phenyl group on the quinoline modulates the physicochemical properties and possibly renders these compounds more cell-permeable. These observations have prompted us to further investigate substitution at the C2 position of the quinoline moiety.

The phenyl ring was replaced with different five-membered ring aromatic heterocycles containing one, two, or three heteroatoms (O, N, and S) as shown in Table 2. Introduction of the various heterocycles shown in Table 2 was well tolerated, producing inhibitors with enzymatic potency comparable to that of starting compound **5**.[23] However, when evaluated in the cell-based replicon assay, a larger range of EC_{50} values were observed. Compound **6**, containing a 5-methyl-1,3,4-oxadiazol-2-yl group, was the least potent analogue in this series, showing an EC_{50} of 200 nM. All other heterocycle-containing compounds shown in Table 2 displayed an improved cell-based potency over **5** and with similar activities in both enzymatic and cellular assays. Pyrrole (**7**) and pyrazole (**8**, **9**) derivatives produced very potent inhibitors, giving rise to compounds with EC_{50} values below 10 nM. Interestingly, two regioisomers, pyrazole derivatives **9** (3.0 nM) and **10** (22 nM) demonstrated the lowest and the highest IC_{50} of this series of compounds with a similar range of inhibitory activities when evaluated in the cell-based replicon assay. Among the best heterocycles identified were the 2-amino-4-thiazolyl derivatives. Compounds **13**−**15** are low-nanomolar inhibitors of the NS3 protease in both enzymatic and cellular assays. The potency of these inhibitors was independent of the substituent on the amino group. From this study, several novel five-membered ring heterocycles were identified as 2-quinoline substituents that produced inhibitors with the target cellular potency.

We then evaluated alternative capping groups. The stability of the *tert*-butyl carbamate capping group was a concern because it is well documented that the *tert*-butyl carbamate is not stable under acidic conditions.[24] Early SAR studies conducted on a related series of acyclic inhibitors (manuscript in preparation) had identified carbamates arising from cyclic alcohols, as well as several ureas, which are well tolerated and impart chemical stability. On the basis of these studies, cyclobutyl (**16**) and cyclopentyl (**17**) carbamates as well as α-methylneopentyl urea (**18**) were introduced in combination with the 2-(2-acetylamino-4-thiazolyl)-quinoline present in **15**. Results are summarized in Table 3.

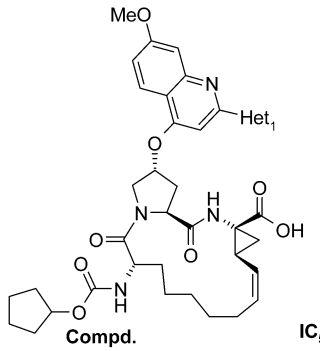
Table 3. Evaluation of Different Capping Groups

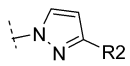
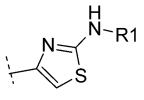
CG	Compd.	IC_{50}(nM)	EC_{50}(nM)
	**15**	4.8	4.5
	**16**	2.8	0.6
	**17**	2.5	0.4
	**18**	1.4	20



We were very pleased to find that introduction of either the cyclobutyl or the cyclopentyl carbamate improved considerably the cell-based potency. Indeed, **16** and **17** were ∼10-fold more potent than analogue **15** and are representative of a group of compounds with subnanomolar potency in the HCV replicon assay. The introduction of a urea capping group (inhibitor **18**) produced the most potent compound in the enzymatic assay; however, considerable loss in potency was observed in the cell-based assay. Perhaps the presence of an additional polar (NH) group decreases cell permeability.[25]

Table 4. Combination of Cyclopentyl Carbamate Capping Group with Optimal 2-Hetorocycle Quinoline



Het_1	Compd.		IC_{50}(nM)	EC_{50}(nM)
	**19**		3.6	3.4
	**20**	R2 = H	2.2	1.1
	21	R2 = Me	2.2	1.2
	**22**	R1 = H	3.5	1.6
	23	R1 = Me	2.5	0.8
	24	R1 = Et	2.9	1.0
	BILN 2061	R1 = *iso*-Pr	3.0	1.2

Encouraged by the beneficial effect of introducing a cyclopentyl carbamate capping group on derivative **15**, we decided to evaluate this capping group with other quinoline derivatives. Table 4 shows the combination of the cyclopentyl carbamate with pyrrole (**19**), pyrazole (**20**, **21**), and aminothiazole-containing derivatives (**22**−**24**, BILN 2061). In all cases, the cell-based potency was improved compared to the *tert*-butyl carbamate analogue. Several compounds with low-nanomolar and subnanomolar potency were identified, increasing the pool of compounds with the target cellular potency.

We then evaluated the pharmacokinetic properties of the best inhibitors in rats. Compounds were administered either orally (20 mg/kg) or intravenously(5 mg/kg).[26] The oral dosing of compounds **8**, **9**, **13**, **17**, **20** (5 mg/kg), **21**, and **22** resulted in undetectable levels in the rat plasma. In contrast, the oral administration of **15** displayed low plasma concentrations (maximum plasma concentration, $C_{max} = 0.3\ \mu M$ and $AUC_{0-\infty} = 0.3\ \mu M \cdot h$) and an improved half-life ($T_{1/2} = 0.8$ h) over **5** after intravenous dosing. The bioavailability of compound **15** is 8%.

A short plasma half-life and a high clearance rate for peptidomimetic compounds have been reported, an example being the HIV protease inhibitors.[27] In an attempt to understand the poor oral bioavailability of some of these compounds, the importance of the biliary excretion to the clearance of **15** was investigated. Following an intravenous bolus injection of 5 mg/kg in rats,[26] **15** was efficiently excreted into the bile unchanged with a recovery of greater than 67% of the dose after 1 h. These results are consistent with a major hepatobiliary elimination and would suggest a high liver first-pass effect after oral dosing.

Compound **24** showed higher plasma levels after oral administration to rats with a maximum plasma concentration of 1 μM and an area under the plasma concentration−time curve of 7.2 $\mu M \cdot h$. Increasing the size of the alkyl group on the 2-aminothiazolyl derivative from ethyl to isopropyl generated BILN 2061 with enzymatic and cell-based inhibitor potencies similar to those of **24**. BILN 2061 showed an improved pharmacokinetic profile (Table 5) with a much higher C_{max} and $AUC_{0-\infty}$ and an oral bioavailability of 42% in rats. On the basis of its excellent in vitro potency and adequate pharmacokinetic profile in rats, BILN 2061 was further evaluated. A satisfactory pharmacokinetic profile in higher species and adequate preclinical safety profile were observed, supporting further evaluation in humans.[17] When BILN 2061 was administered orally to patients infected with HCV genotype 1 for 2 days, an unprecedented (up to 3 log) decline in plasma viral load was observed. This represented the first proof-of-concept in man for an HCV NS3 protease inhibitor. A full description of the biopharmaceutical profile of BILN 2061 has been reported elsewhere.[17]

Table 5. Pharmacokinetic Parameters for BILN 2061 in Rats Following Oral and Intravenous Administration

oral, 20 mg/kg		iv, 5 mg/kg			
C_{max} (μM)	$AUC_{0-\infty}$ ($\mu M \cdot h$)	$T_{1/2}$ (h)	V_{ss} (L/kg)	Cl ((mL/min)/kg)	F (%)
2.5	12.5	1.3	1.0	13	42

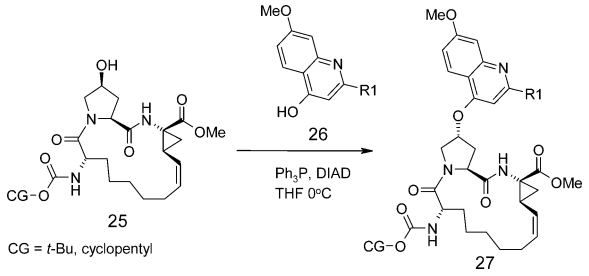
All the inhibitors shown in this letter contain a C-terminal carboxylic acid that renders them very potent and specific for the NS3 protease.[19] These compounds do not significally inhibit human serine/cysteine proteases such as human leukocyte elastase and cathepsin S ($IC_{50} > 30\ \mu M$).

Summary. Focused structure−activity studies on the tripeptide macrocyclic inhibitor **5** led to the discovery of BILN 2061, a very potent and specific inhibitor of the HCV NS3 protease in both the enzymatic ($IC_{50} = 3.0$ nM) and the cell-based replicon assays ($EC_{50} = 1.2$ nM). The compound shows good oral bioavailability in rats ($F = 42\%$) with a low plasma clearance (Cl = 13 (mL/min)/kg). BILN 2061 was selected for further development.

Synthesis. The synthesis of these compounds was previously described in detail,[28] and a general approach is shown in Scheme 1. Appropriately, 2-substituted 4-hydroxy-7-methoxyquinolines **26** are coupled with a preformed macrocyclic tripeptide via a Mitsunobu reaction.[29] The resulting methyl esters **27** were hydrolyzed under basic conditions after appropriate adjustments of the capping group.

The synthesis of the various C2-substituted-4-hydroxyquinolines **26** was done on an individual basis and is described in detail in the Supporting Information.

Scheme 1. Inhibitor Assembly



Acknowledgment. The authors thank Dr. Lisette Lagacé, Ms. Lyne Lamarre, Ms. Diane Thibeault, and Ms. Andrea Gorys for EC_{50} and IC_{50} determinations. We also thank the analytical chemistry department for technical support and Ms. Christiane Yoakim and Dr. Janice Kelland for proofreading the manuscript. Finally, we thank Dr. Paul Anderson, Dr. Michael Bös, and Dr. Michael Cordingley for encouragement and support.

Supporting Information Available: ^{1}H NMR, HPLC, and HRMS data for all the compounds disclosed in this letter and a detailed synthetic scheme for the various C2-substituted 4-hydroxyquinolines **26**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Choo, Q.-L.; Kuo, G.; Weiner, A. J.; Overby, L. R.; Bradley, D. W.; Houghton, M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome *Science* **1989**, *244*, 359.

(2) Hagedorn, C. H.; Rice, C. M. The hepatitis C virus. *Curr. Top. Microbiol. Immunol.* **2000**, *242*.

(3) World Health Organization: Hepatitis C. Seroprevalence of hepatitis C virus (HCV) in a population sample. *Wkly Epidemiol. Rec.* **1996** *71*, 346−349.

(4) Cornberg, M.; Wedemeyer, H.; Manns, M. P. Treatment of chronic hepatitis C with PEGylated interferon and rivavarin. *Curr. Gastroenterol. Rep.* **2002**, *4*, 23−30.

(5) Reed, K. E.; Rice, C. M. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Curr. Top. Microbiol. Immunol.* **2000**, *242*, 55−84.

(6) Beaulieu, P. L.; Llinàs-Brunet, M. Therapies for Hepatitis C Infection: Targeting the Non-Structural Proteins of HCV. *Curr. Med. Chem.: Anti-Infect. Agents* **2002**, *1*, 163−176.

(7) Lesk, A. M.; Fordham, W. D. Conservation and variability in the structure of serine proteinases of the chymotrypsin family. *J. Mol. Biol.* **1996**, *258*, 501−537.

(8) Steinkuhler, C.; Koch, U.; Narjes, F.; Matassa, V. G. Hepatitis C Virus Serine Protease Inhibitors: Current Progress and Future Challenges. *Curr. Med. Chem.* **2001**, *8*, 919−932.

(9) Kolykhalov, A. A.; Mihalik, K.; Feinstone, S. M.; Rice, C. M. Hepatitis C Virus-Encoded Enzymatic Activities and Conserved RNA Elements in the 3′ Nontranslated Region Are Essential for Virus Replication in Vivo. *J. Virol.* **2000**, *74*, 2046.

(10) Llinàs-Brunet, M.; Bailey, M.; Fazal, G.; Goulet, S.; Halmos, T.; Laplante, S.; Maurice, R.; Poirier, M.; Poupart, M.-A.; Thibeault, D.; Wernic, D.; Lamarre, D. Peptide-based inhibitors of the hepatitis C virus serine protease. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1713−1718.

(11) Steinkühler, C.; Biasiol, G.; Brunette, M.; Urbani, A.; Koch, U.; Cortese, R.; Pessi, A.; De Francesco, R. Product inhibition of the hepatitis C virus NS3 protease. *Biochemistry* **1998**, *37*, 8899−8905.

(12) Llinàs-Brunet, M.; Bailey, M.; Fazal, G.; Ghiro, E.; Gorys, V.; Goulet, S.; Halmos, T.; Maurice, R.; Poirier, M.; Poupart, M.-A.; Rancourt, J.; Thibeault, D.; Wernic, D.; Lamarre, D. Highly potent and selective peptide-based inhibitors of the hepatitis C virus serine protease: towards smaller inhibitors. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2267−2270.

(13) Pause, A.; Kukolj, G.; Bailey, M.; Brault, M.; Dô, F.; Halmos, T.; Lagacé, L.; Maurice, R.; Marquis, M.; McKercher, G.; Pellerin, C.; Pilote, L.; Thibeault, D.; Lamarre, D. An NS3 serine protease inhibitor abrogates replication of subgenomic hepatitis C virus RNA. *J. Biol. Chem.* **2003**, *278*, 20374−20380.

(14) Goudreau, N.; Cameron, D. R.; Bonneau, P.; Gorys, V.; Plouffe, C.; Poirier, M.; Lamarre, D.; Llinàs-Brunet, M. NMR Structural Characterization of Peptide Inhibitors Bound to the HCV NS3 Protease: Design of a New P2 Substituent. *J. Med. Chem.* **2004**, *47*, 123−132.

(15) Rancourt, J.; Cameron, D.; Gorys, V.; Lamarre, D.; Poirier, M.; Thibeault, D.; Llinàs-Brunet, M. Peptide-Based Inhibitors of the Hepatitis C Virus NS3 Protease: Structure−Activity Relationship at the C-Terminal Position. *J. Med. Chem.*, in press.

(16) Tsantrizos, Y. S.; Bolger, G.; Bonneau, P.; Cameron, D. R.; Goudreau, N.; Kukolj, G.; LaPlante, S. R.; Llinàs-Brunet, M.; Nar, H.; Lamarre, D. Macrocyclic inhibitors of the NS3 protease as potential therapeutic agents of hepatitis C virus infection. *Angew. Chem., Int. Ed.* **2003**, *42*, 1356−1360.

(17) Lamarre, D.; Anderson, P.; Bailey, M.; Beaulieu, P.; Bolger, G.; Bonneau, P.; Bös, M.; Cameron, D.; Cartier, M.; Cordingley, M.; Faucher, A.-M.; Goudreau, N.; Kawai, S.; Kukolj, G.; Lagacé, L.; LaPlante, S.; Narjes, H.; Poupart, M.-A.; Rancourt, J.; St-George, R.; Sentjens, R. E.; Simoneau, B.; Steinmann, G.; Thibeault, D.; Tsantrizos, Y.; Weldon, A. M.; Yong, C.-L.; Llinàs-Brunet, M. Discovery of BILN 2061−An NS3 preotease inhibitors with first antiviral proof-of-concept in humans infected with hepatitis C virus. *Nature* **2003**, *426*, 186−189.

(18) Tsantrizos, Y. S.; et al. Manuscript in preparation.

(19) Llinàs-Brunet, M.; Bailey, M.; Déziel, R.; Fazal, G.; Gorys, V.; Goulet, S.; Halmos, T.; Maurice, R.; Poirier, M.; Poupart, M.-A.; Rancourt, J.; Thibeault, D.; Wernic, D.; Lamarre, D. l. Studies on the C-terminal of hexapeptide inhibitors of the hepatitis C virus serine protease. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2719−2724.

(20) Poupart, M.-A.; Cameron, D. R.; Chabot, C.; Ghiro, E.; Goudreau, N.; Goulet, S.; Poirier, M.; Tsantrizos, Y. Solid-phase synthesis of peptidomimetic inhibitors for the hepatitis C virus NS3 protease. *J. Org. Chem.* **2001**, *66*, 4743−4751.

(21) LaPlante, S. R.; Cameron, D. C.; Aubry, N.; Lefebvre, S.; Kukolj, G.; Maurice, R.; Thibeault, D.; Lamarre, D.; Llinàs-Brunet, M. Solution structure of substrate-based ligands when bound to hepatitis C virus NS3 protease domain. *J. Biol. Chem.* **1999**, *274*, 18618−18624.

(22) LaPlante, S. R.; Aubry, N.; Bonneau, P. R.; Kukolj, G.; Lamarre, D.; Lefebvre, S.; Li, H.; Llinàs-Brunet, M.; Plouffe, C.; Cameron, D. R. NMR line-broadening and transferred NOESY as a medicinal chemistry tool for studying inhibitors of the hepatitis C virus NS3 protease domain. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2271−2274.

(23) The values reported in both the enzymatic (IC_{50}) and cell-based assay (EC_{50}) were determined using the assays described in ref 13. The values reported are an average of at least four determinations.

(24) Greene, T. W., Wuts, P. G. M., Eds. *Protective Groups in Organic Synthesis*, 3rd ed.; John Wiley & Sons: New York, 1999; p 518.

(25) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *1−3*, 3−25.

(26) Rats were administered compounds as either an oral gavage or intravenous bolus dose in a vehicle consisting of PEG400/water (70:30). Protocols for the experiments are described in the supporting information of ref 16.

(27) Denissen, J. F.; Grabowski, B. A.; Johnson, M. K.; Buko, A. M.; Kempf, D. J.; Thomas, S. B.; Surber, B. W. Metabolism and disposition of the HIV-1 protease inhibitors Ritonavir (ABT-538) in rats, dogs and humans. *Drug Metab. Dispos.* **1995**, *23*, 185−189.

(28) Tsantrizos, Y. S.; Cameron, D.; Faucher, A.-M.; Ghiro, E.; Goudreau, N.; Halmos, T.; Llinàs-Brunet, M.; Boehringer Ingelheim (Canada) Ltd. Macrocyclic peptides active against the Hepatitis C virus. U.S. Patent 6,608,027 B1, 2003.

(29) Yoakim, C.; Guse, I.; O'Meara, J. A.; Thavonekham, B. Removable Phosphine Reagents for the Mitsunobu Reaction. *Synlett* **2003**, 473−476.

JM0342414