Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 701–704

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A Designed P_1 Cysteine Mimetic for Covalent and Non-Covalent Inhibitors of HCV NS3 Protease

Frank Narjes,[a,*] Konrad F. Koehler,[a] Uwe Koch,[a] Benjamin Gerlach,[a] Stefania Colarusso,[a] Christian Steinkühler,[b] Mirko Brunetti,[b] Sergio Altamura,[b] Raffaele De Francesco[b] and Victor G. Matassa[a]

[a]*Department of Chemistry, IRBM, MRL Rome, Via Pontina Km 30.600, Pomezia, 00040 Rome, Italy*
[b]*Department of Biochemistry, IRBM, MRL Rome, Via Pontina Km 30.600, Pomezia, 00040 Rome, Italy*

Received 3 October 2001; revised 4 December 2001; accepted 12 December 2001

Abstract—The difluoromethyl group was designed by computational chemistry methods as a mimetic of the canonical P_1 cysteine thiol for inhibitors of the hepatitis C virus NS3 protease. This modification led to the development of competitive, non-covalent inhibitor **4** (K_i 30 nM) and reversible covalent inhibitors (**6**, K_i 0.5 nM; and **8** K_i* 10 pM).

The hepatitis C virus (HCV) is the major causative agent of parenterally-transmitted and sporadic non-A, non-B hepatitis (NANB-H),[1] and is believed to affect some 200 million people worldwide.[2] Infection by the virus can result in chronic hepatitis and cirrhosis of the liver, and may lead to hepatocellular carcinoma. There is neither a vaccine nor an established therapy for HCV, although partial success has been achieved by treatment with recombinant α-interferon, either alone or in combination with ribavirin.[2,3]

A serine protease located in the N-terminal domain of the NS3 protein is one of the prime targets for the development of antiviral drugs, since it is responsible for proteolytic maturation of most of the non-structural region of the viral polyprotein. The NS3 protease belongs to the trypsin superfamily, and is unique in requiring a (non-catalytic) structural zinc atom and a co-factor protein (NS4A).[4,5] Crystal structures of the protease[4,5] reveal that the S (non-prime)[6] site of the substrate-binding channel is strongly cationic, solvent-exposed and relatively featureless, explaining why the minimum substrate is a polycarboxy decapeptide.

The NS3 protease is subject to product inhibition by N-terminal hexapeptide[7] and tetrapeptide[8] carboxylic acids. The reversible competitive inhibitor **1** (K_i 40 nM, Table 1) emerged from optimization of an initial lead identified in biochemical studies. Cysteine was established as the preferred P_1 amino acid for intermolecular cleavage of substrate by NS3 and for product-based inhibitors.[5] This structural characteristic complicates the ready preparation of serine-trap inhibitors due to the juxtaposition of mutually-incompatible nucleophilic (i.e., thiol) and electrophilic (i.e., serine-trap) functionality. As the thiol is a potential Achilles' heel for any drug that derives from this series of molecules, we sought a surrogate for the P_1 cysteine sulfhydryl to facilitate the preparation of stable biochemical tools and to progress our drug discovery efforts. We report here: (i) the design by computational chemistry methods of a P_1 thiol replacement; (ii) its successful introduction into product-based inhibitors; and (iii) its utility in preparing potent serine-trap inhibitors of NS3. In the accompanying paper we will present the evolution of these inhibitors to small and potent tripeptides.[9]

Design of a P_1 Thiol Replacement

The S_1 specificity pocket of the NS3 protease is small and lipophilic, comprising Leu135, Phe154 and Ala157. Substrate mutagenesis studies[4,5] and product inhibitor optimization[7] concur that cysteine is preferred at P_1. The cysteine sulfhydryl is lipophilic, but has lone-pairs

*Corresponding author. Tel.: +39-06-910-93-625; fax: +39-06-910-93-654; e-mail: frank_narjes@merck.com

0960-894X/02/$ - see front matter
PII: S0960-894X(01)00842-3

of electrons and a polarized S–H bond which confer the potential for electrostatic non-covalent interactions.[10] In attempting to discover a novel P_1 substituent, an analysis was therefore made of the steric and electrostatic properties of the thiol.[11] Subsequent comparison with a selection of small fluoroalkyl and fluoroalkenyl groups suggested interesting parallels for CF_2H. The steric and electrostatic parameters for CF_2H and SH were evaluated on 1,1-difluoroethane and methanethiol, respectively. The volume of 1,1-difluoroethane, 46.7 Å^3, is similar to that of methanethiol (47.1 Å^3) (Fig. 1). The MEP surfaces are also similar (Fig. 2), displaying a substantial build-up of negative potential around the sulfur lone pairs and the two fluorine atoms, and a positive potential around the S- and CF_2-bound protons. The CF_2H and SH groups thus emerged as having similar calculated steric and electrostatic features which encouraged an investigation of CF_2H in the P_1 position of NS3 protease inhibitors.

Synthesis

The synthesis of **1**, **2**, **8** and **9** have been described.[7,12] The aldehyde **6** was prepared as outlined in Scheme 1.

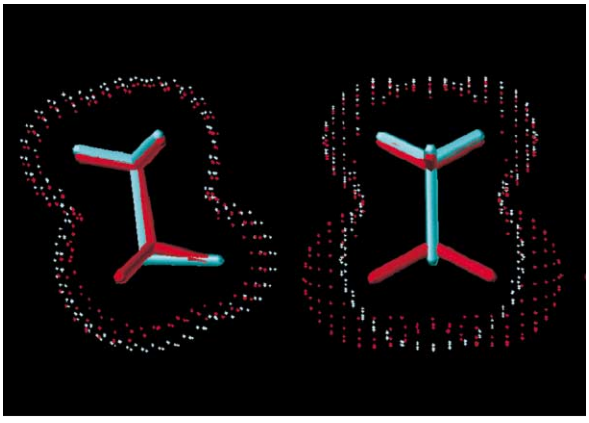


Figure 1. Orthogonal views of the van der Waals surfaces of methanethiol (cyan) and 1,1-difluoroethane (red). The surfaces are Z-clipped for clarity.

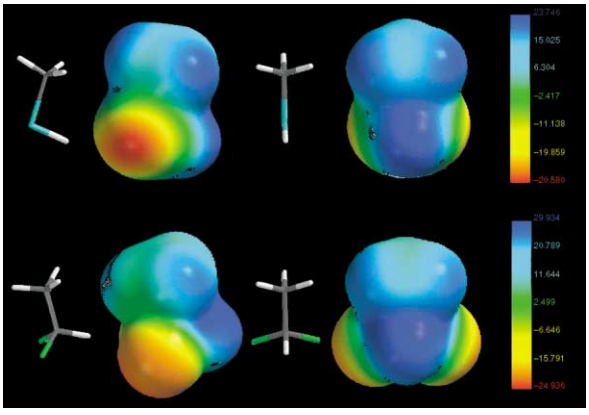


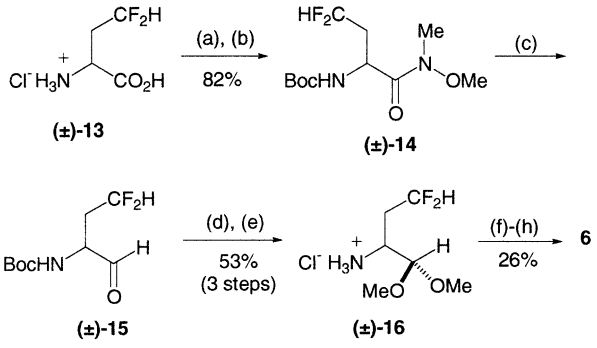
Figure 2. Orthogonal views of electrostatic potentials displayed over the electron density surface of methanethiol (top) and 1,1-difluoroethane (bottom). Stick diagrams of the respective compounds are displayed to the left and color-coded scales of electrostatic potential are displayed on the right for reference.

Thus, (±)-4,4-difluoro-2-aminobutyric acid (difluoroAbu) (**13**)[13] was converted to the aldehyde **15** in three steps. Acetalization and removal of the Boc group gave amine **16**. Coupling with the pentapeptide AcAsp(O*t*Bu)Glu(O*t*Bu)DifGlu(O*t*Bu)-ChaOH (**17**) was followed by complete deprotection and purification with separation of the diastereomers by HPLC to give the aldehyde **6**.[14] Product inhibitors **4** and **5** were synthesized as described[12] using chiral **13** prepared from L- or D-aspartic acid.[15] Using allylglycine instead of difluoroAbu gave inhibitors **3** and **7**.[16]

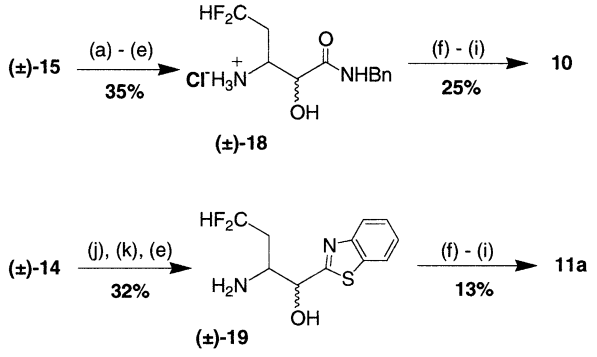
Scheme 2 shows the synthesis of α-ketoamide **10** and the α-ketoheterocycles **11**–**12**, as exemplified for benzothiazole **11a**. Conversion of aldehyde **15** to the α-hydroxyamide **18** was achieved in five steps, while reaction of Weinreb amide **14** with 2-lithio-benzothiazole gave **19** after reduction and deprotection. Both compounds were then converted to the final products as described above, except that oxidation of the hydroxy groups was carried out prior to the final deprotection step.

Results and Discussion

Substitution of the P_1 cysteine by (*S*)-difluoroAbu was first investigated in hexapeptide product acids. Inhibitors were evaluated on the NS3/4A complex described



Scheme 1. (a) Boc_2O; (b) MeNH(OMe) HCl, EDCI, HOBt, *i*Pr_2NEt; (c) DIBAL, THF, −78 °C; (d) $HC(OMe)_3$, TsOH; (e) HCl, MeOH, 0 °C–rt; (f) **17**, EDCI, HOBt, *i*Pr_2NEt, DCM; (g) TFA, H_2O, DCM; (h) RP-HPLC.

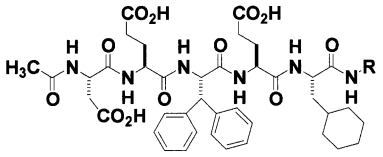


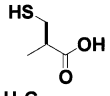
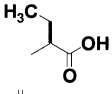
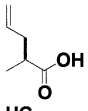
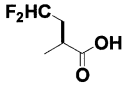
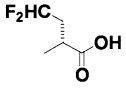
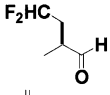
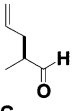
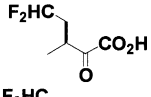
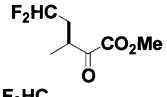
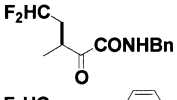
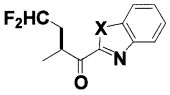
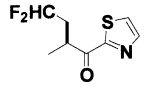
Scheme 2. (a) $Me_2C(OH)CN$, TEA, DCM; (b) HCl, H_2O, dioxane; (c) Boc_2O; (d) $BnNH_2$, EDCI, HOBt; (e) HCl, EtOAc; (f) **17**, EDCI, HOBt, *i*Pr_2NEt, DCM; (g) Dess–Martin, DCM, *t*-BuOH; (h) TFA, H_2O, DCM; (i) RP-HPLC; (j) benzothiazole, BuLi, THF, −78 °C; (k) $NaBH_4$, EtOH, 0 °C.

previously.[17] The difluoro analogue **4** (K_i 30 nM) is at least as potent as its cysteine counterpart **1** (K_i 40 nM)[7] and substantially more active than the Abu and the allyl analogues **2** and **3** (Table 1). Compound **5** (K_i 640 nM), the P_1 diastereomer of **4,** was less active.

The identification of this non-reactive and equipotent thiol surrogate facilitated the synthesis and evaluation of serine trap-based inhibitors. Of these the α-ketoacid **8** (K_i* 10 pM) proved to be particularly effective. Kinetic investigations showed that the potency of **8** is due to the extremely slow dissociation rate of the covalent adduct.[12] The aldehyde **6** (K_i 0.5 nM) is a reversible inhibitor whose affinity is intermediate between that of the product acid **4** and the ketoacid **8**. Allyl aldehyde **7** was significantly less active than **6**.

Table 1. Inhibitors of NS3/4A protease



Compd	R	*Ki* (nM)
1		40
2		700
3		100
4		30
5		640
6		0.5
7		7
8		0.01*
9		2.0
10		1.5
11a (X = S) **11b** (X = O)		150 600
12		1500

Simplistically, ketoacid **8** may be considered as an inhibitor that combines the binding energy contributed by a covalent bond (as in aldehyde **6**) with the electrostatic stabilization of the charged inhibitor by Lys136 (as in acid **4**).[12,18] This is also evident from two close analogues, α-ketoester **9** and α-ketoamide **10**. Although both are potent inhibitors of NS3, as was observed previously in related series,[19] they do not achieve the potency of **8**.

Inhibitors incorporating the α-keto heterocycle moiety like **11** and **12** proved to be less active than **4**, **7**, or **8**. These heterocyclic groups give potent inhibitors with the related proteases elastase, chymase and thrombin.[20] Presumably, their lack of activity is due to some steric restriction in the active site of the enzyme, which prevents optimal binding.

From our results and previous studies, it is evident that the substitution of the P_1 cysteine using conventional amino acids in inhibitors and in substrates failed to find an effective replacement,[4,5,17] suggesting that the difluoromethyl group has specific properties which mimic the thiol. The CF_2H group has a similar volume and shape to SH (Fig. 1). Also, SH and CF_2H are lipophilic, a feature that is presumably of importance to binding, since the P_1 pocket is substantially hydrophobic.

Electrostatic properties could play a role, however. The P_1 cysteine has non-covalent contacts with the phenyl ring of the S_1 Phe154[4,5] (Fig. 3). The thiol could interact with the phenyl ring either as a proton donor, through interaction of the SH proton with the electron-rich *pi*-cloud, or as a proton acceptor, through interaction of the sulfur lone electron pairs with a ring proton, the latter having partial positive character. Electrostatic interactions involving the *pi*-cloud and protons of a phenyl ring are well documented.[21] Directional sulfur-aromatic interactions are commonly observed in protein crystal structures,[22] and the thiol group can be a hydrogen bond donor or acceptor in biological systems.[23,24] The cysteine sulfhydryl thus could in principle act in either capacity in the NS3 protease P_1–S_1 interaction. Despite its calculated electron-richness, in practice aliphatic fluorine rarely functions as a hydrogen

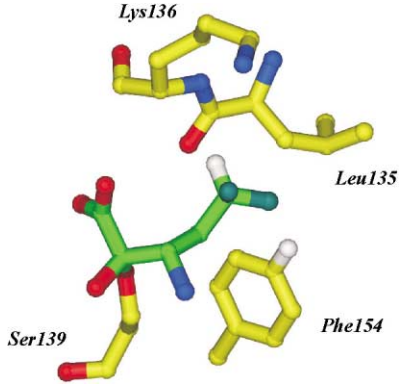


Figure 3. Interactions of the P_1 difluoroAbu in the P_1 specificity pocket according to the crystal structure of the NS3-protease with the ketoacid covalently bound to the catalytic serine 139.

bond acceptor, probably because of its high electronegativity and low polarizability.[25,26] In contrast, CF_2H has been documented as a proton *donor* in hydrogen bonding interactions.[27] Recent structural studies[28] of tripeptide analogues of **6** co-crystallized with NS3/4A confirm this design. The CF_2H moiety is almost completely buried in the P_1-specificity pocket creating a large lipophilic contact surface area. In this structure the flexible, lipophilic part of the Lys136 sidechain is oriented so that it covers the CF_2H moiety, while the ammonium group is close to the ketoacid carboxylate (Fig. 3). In the rather apolar environment of the P_1-specificity pocket electrostatic complementarity becomes important, even for relatively unpolar groups. Accordingly, the CF_2H is oriented with its proton close to the carbonyl oxygen of Lys136 and one fluorine atom in contact with the *para* hydrogen of Phe154. Thus, steric and electrostatic similarity of the Cys sidechain and difluoroAbu allows a similar mode of binding in the P_1-specificity pocket with almost complete burial of the sidechain. The observation that difluoroAbu works under such highly specific conditions as a Cys mimetic underlines its general applicability as a non-reactive Cys surrogate.

Acknowledgements

The authors thank K. Prendergast for valuable discussions, B. Wang for early contributions, A. Pessi for a sample of **2**, S. Pesci for NMR spectra, and F. Naimo and F. Bonelli for Mass spectra.

References and Notes

1. Review: Clarke, B. *J. Gen. Virol.* **1997**, *78*, 2397.
2. Cohen, J. *Science* **1999**, *285*, 26.
3. Dymock, B. W.; Jones, P. S.; Wilson, F. X. *Antiviral Chem. Chemother.* **2000**, *11*, 79.
4. Review: Kwong, A. D.; Kim, J. L.; Rao, G.; Lipovsek, D.; Raybuck, S. A. *Antiviral Res.* **1998**, *40*, 1.
5. Review: De Francesco, R.; Steinkühler, C. *Curr. Top. Micr. Immunol.* **2000**, *242*, 149.
6. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
7. Ingallinella, P.; Altamura, S.; Bianchi, E.; Taliani, M.; Ingenito, R.; Cortese, R.; De Francesco, R.; Steinkühler, C.; Pessi, A. *Biochemistry* **1998**, *37*, 8906.
8. 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. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2267.
9. Colarusso, S.; Gerlach, B.; Koch, U.; Muraglia, E.; Conte, I.; Stansfield, I.; Matassa, V. G.; Narjes, F. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 705.
10. Burley, S. K.; Petsko, G. A. In Weakly Polar Interactions in Proteins. In: *Adv. Prot. Chem.* 39; Anfinsen, C. B., Edsall, J. T., Richards, F. M., Eisenberg, D. S., Eds.; Academic: San Diego, USA, 1988; p 125.
11. Calculations were performed on a Silicon Graphics Extreme workstation (R4000 CPU). Ab initio calculations were performed using Spartan version 5.02, with default settings. Starting geometries of methanethiol and 1,1-difluoroethane were built using the Spartan molecular editor and were fully optimized using the 6-31G* basis set. Molecular electrostatic potentials (MEP) were plotted over the total electron density surface using the 6-31G* wave function. Ab initio optimized geometries were imported into Sybyl version 6.3 and the surfaces were generated using the Van der Waals dot option.
12. Narjes, F.; Brunetti, M.; Colarusso, S.; Gerlach, B.; Koch, U.; Biasiol, G.; Fattori, D.; De Francesco, R.; Matassa, V. G.; Steinkühler, C. *Biochemistry* **2000**, *39*, 1849.
13. Analogous to the trifluoro analogue: Tsushima, T.; Kawada, K.; Ishihara, S.; Uchida, N.; Shiratori, O.; Higaki, J.; Hirata, M. *Tetrahedron* **1988**, *44*, 5375.
14. The more active diastereomer was assigned the L-configuration at P_1, in accord with the results from **4** and **5** and recently published structures of enzyme inhibitor complexes (see ref 28).
15. Winkler, D.; Burger, K. *Synthesis* **1999**, 1419.
16. Intermediates and final compounds have been characterized by ^1H and ^{13}C NMR, MS and RP-HPLC.
17. Urbani, A.; Bianchi, E.; Narjes, F.; Tramontano, A.; De Francesco, R.; Steinkühler, C.; Pessi, A. *J. Biol. Chem.* **1997**, *272*, 9204.
18. Koch, U.; Biasiol, G.; Brunetti, M.; Fattori, D.; Brunetti, M.; Palloro, M.; Steinkühler, C. *Biochemistry* **2001**, *40*, 631.
19. (a) Bennett, J. M.; Campbell, A. D.; Campbell, A. J.; Carr, M. G.; Dunsdon, R. M.; Greening, J. R.; Hurst, D. N.; Jennings, N. S.; Jones, P. S.; Jordan, S.; Kay, P. B.; O'Brien, M. A.; King-Underwood, J.; Raynham, T. M.; Wilinson, C. S.; Wilinson, T. S. I.; Wilson, F. X. L. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 355. (b) Han, W.; Hu, Z.; Jiang, X.; Decicco, C. P. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 711.
20. Akahoshi, F.; Ashimori, A.; Sakashita, H.; Yoshimura, T.; Imada, T.; Nakajima, M.; Mitsutomi, N.; Kuwahara, S.; Ohtsuka, T.; Fukaya, C.; Miyazaki, M.; Nakamura, N. *J. Med. Chem.* **2001**, *44*, 1286 and references cited therein.
21. Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303 and references cited therein.
22. Reid, K. S. C.; Lindley, P. F.; Thornton, J. M. *FEBS* **1985**, *190*, 209.
23. Platts, J. A.; Howard, S. T.; Bracke, B. R. F. *J. Am. Chem. Soc.* **1996**, *118*, 2726.
24. Erlanson, D. A.; Verdine, G. L. *Chem. Biol.* **1994**, *1*, 79.
25. Dunitz, J. D.; Taylor, R. *Chem. Eur. J.* **1997**, *3*, 89.
26. Howard, J. A. K.; Hay, V. J.; O'Hagan, D.; Smith, G. T. *Tetrahedron* **1996**, *52*, 12613.
27. Erickson, J. A.; McLoughlin, J. *J. Org. Chem.* **1995**, *60*, 1626.
28. Di Marco, S.; Rizzi, M.; Volpari, C.; Walsh, M. A.; Narjes, F.; Colarusso, S.; De Francesco, R.; Matassa, V. G.; Sollazzo, M. *J. Biol. Chem.* **2000**, *275*, 7152.