Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2355–2359

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Structure–Activity Relationship Studies of a Bisbenzimidazole-Based, Zn^{2+}-Dependent Inhibitor of HCV NS3 Serine Protease

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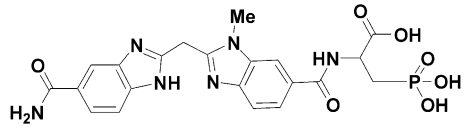
Received 6 April 2001; accepted 21 June 2001

Abstract—A survey of isosteric replacements of the phosphonoalanine side chain coupled with a process of conformational constraint of a bisbenzimidazole-based, Zn^{2+}-dependent inhibitor of hepatitis C virus (HCV) NS3 serine protease resulted in the identification of novel series of active compounds with extended side chains. However, Zn^{2+}-dependent HCV NS3 inhibition was relatively insensitive to the structural variations examined but dependent on the presence of negatively charged functionality. This result was interpreted in the context of an initial electrostatic interaction between protease and inhibitor that is subsequently consolidated by Zn^{2+}, with binding facilitated by the featureless active site and proximal regions of the HCV NS3 protein.

Hepatitis C virus (HCV) is the major etiological agent of non-A, non-B hepatitis that was identified in 1989.[1] It has been estimated that 1% of the global population is infected with HCV, affecting approximately 4 million people in the USA. A chronic HCV infection develops in 75% of these individuals and has been linked to the development of liver cirrhosis and hepatocellular carcinoma. At present, the development of an effective vaccine remains an uncertain prospect and current therapy for HCV disease using interferon in combination with ribavirin is less than optimal due to toxicity and low long term response rates.[2]

During the replication of HCV, proteolytic processing of the nonstructural (NS) proteins at four specific sites (NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B) is carried out by a protease located within the amino terminal of nonstructural protein 3 (NS3).[3] The HCV NS3 protease has been determined to be a serine protease with a chymotrypsin/trypsin-like fold that possesses a Ser-His-Asp catalytic triad. It has been established that enzymatic activity is markedly enhanced by a cofactor, NS4A.[3] Since the HCV NS3 serine protease plays an essential role in viral replication, it represents an important drug discovery target that has been the subject of intensive study in the search for efficacious anti-HCV drugs.[2–4]

As part of an effort directed towards the discovery of small molecule inhibitors of HCV NS3 protease, we sought to take advantage of a serine protease inhibitor chemotype in which inhibition is dependent on the formation of a ternary complex between enzyme, inhibitor, and Zn^{2+}.[5] Panning of a library of bisbenzimidazole derivatives as potential inhibitors of HCV NS3 identified the phosphonoalanine derivative **1** (APC-6336) as a potent inhibitor of the protease that displayed an IC_{50} of 0.20 μM in the presence of Zn^{2+}.[6] In the absence of Zn^{2+}, APC-6336 (**1**) was over 800-fold less active with an IC_{50} of 167 μM (Table 1).[7]



1 (APC-6336)

It was envisioned that the interaction of APC-6336 (**1**) with HCV NS3 was such that the bisbenzimidazole core occupied the active site of the enzyme and, together with the catalytic serine hydroxyl and histidine imidazole acting as the other two ligands, formed a tetrahedral

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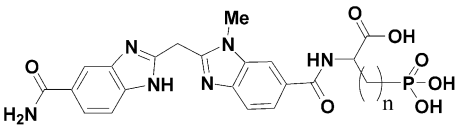
0960-894X/01/$ - see front matter
PII: S0960-894X(01)00457-7

Table 1. Assay results for compounds **1**–**4** against HCV NS3 protease domain [amino acids (aa) 1–183] in the presence of Zn^{2+}[7]



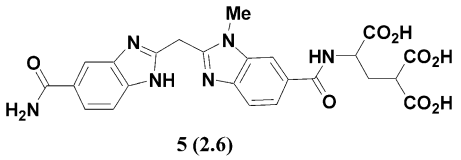
Compound	*n*	IC_{50} (μM)
1 (APC-6336)	1	0.20[a]
2	2	0.87
3	3	0.82
4	5	0.51

[a]IC_{50} = 167 μM in the absence of Zn^{2+} and the presence of EDTA.

coordination complex with a Zn^{2+} atom.[5] As a consequence of the pseudo symmetrical topology, the phosphonoanaline moiety could project into either the P or P′ sites.

As one aspect of the development of SAR for this series of HCV NS3 protease inhibitors, interactions of the phosphonoalanine side chain of APC-6336 (**1**) with the protease were examined in a broad fashion by a process of isosteric replacement and side-chain rigidification with simultaneous introduction of hydrophobicity, a strategy that also served as an avenue to identify new points of contact between inhibitor and protease. It was anticipated that the results of these studies would lead to the design of a new generation of non-metal-mediated inhibitors that possessed more desirable structural features, particularly reduced anionic character.

A survey of phosphonoalanine homologues was conducted initially in order to determine the effect of varying carbon chain length on the potency of compound **1**. The results of this study are summarized in Table 1 and reveal that sub-micromolar, Zn^{2+}-dependent inhibitory activity was largely maintained as the chain length was increased. The fully extended AP-7 derivative **4** is only about 2-fold less active than prototype **1** despite a marked increase in conformational flexibility. Indeed, the activity may be retained as a consequence of the structural mobility of the side chain. However, replacing the phosphoalanine moiety with either aspartate or glutmate produced compounds that were over 200-fold less potent than **1**. Since a *gem*-dicarboxyl group may more effectively mimic the doubly charged phosphonate moiety, the γ-carboxyglutamic acid analogue **5** was synthesized and found to exhibit significant inhibitory activity with an IC_{50} of 2.6 μM in the presence of Zn^{2+} (Fig. 1).

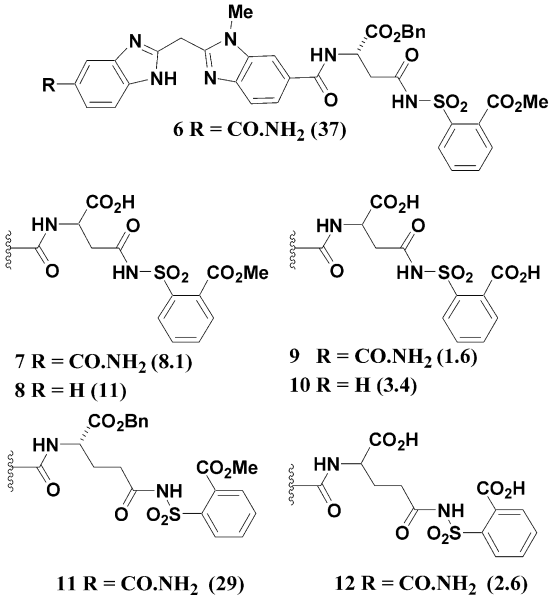


5 (2.6)

Figure 1. Compound **5** and, in parentheses, the IC_{50} (μM) as an inhibitor of the HCV NS3 protease domain (aa 1–183) in the presence of Zn^{2+}.[7]

This theme was further developed by examining the series of amino acid sulfonamides depicted in Figure 2. Analogues **9**, **10**, and **12** demonstrated similar inhibitory activity to **5**, presumably because the acidic acylsufonamide and attendant *ortho* carboxylate together performed as an effective phosphonate mimic. However, the mono methyl esters **7** and **8** retain significant inhibitory properties, suggesting that non-charged polarity is sufficient in this context, but diesters **6** and **11** are notably less effective although still detectably active. Comparing the results for **9** and **10** indicates that the $CONH_2$ moiety distal to the amino acid element does not contribute significantly to inhibitory activity whilst the data recorded for **9** and **12**[8] reveal little dependence on the separation of the charged terminus and the heterocyclic nucleus. The iminodiacetic acid[9] derivative **13**, in which the dicarboxylate moiety is conformationally more flexible and based on a less hydrophobic scaffold, is only 2-fold less potent than **5**.

Further studies focused on exploring a combination of conformational constraint within the context of an extended side chain coupled with phosphonate replacements as a means of enhancing potency. Tyrosyl acetic acid and its carboxylated analogues have been used as phosphonate isosteres[10] and were selected as convenient platforms with which to explore the presentation of acidic functionality, as summarized in Figure 3. From this series, the simple tyrosine-*O*-acetic acid analogue **17** emerged as the most potent compound, IC_{50} = 1 μM in the presence of Zn^{2+}. However, the introduction of



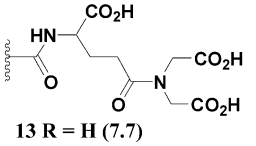
6 R = CO.NH$_2$ (37)

7 R = CO.NH$_2$ (8.1)
8 R = H (11)

9 R = CO.NH$_2$ (1.6)
10 R = H (3.4)

11 R = CO.NH$_2$ (29)

12 R = CO.NH$_2$ (2.6)



13 R = H (7.7)

Figure 2. Compounds **6**–**13** and, in parentheses, their IC_{50} (μM) as inhibitors of HCV NS3 protease domain (aa 1–183) in the presence of Zn^{2+}.[7,8]

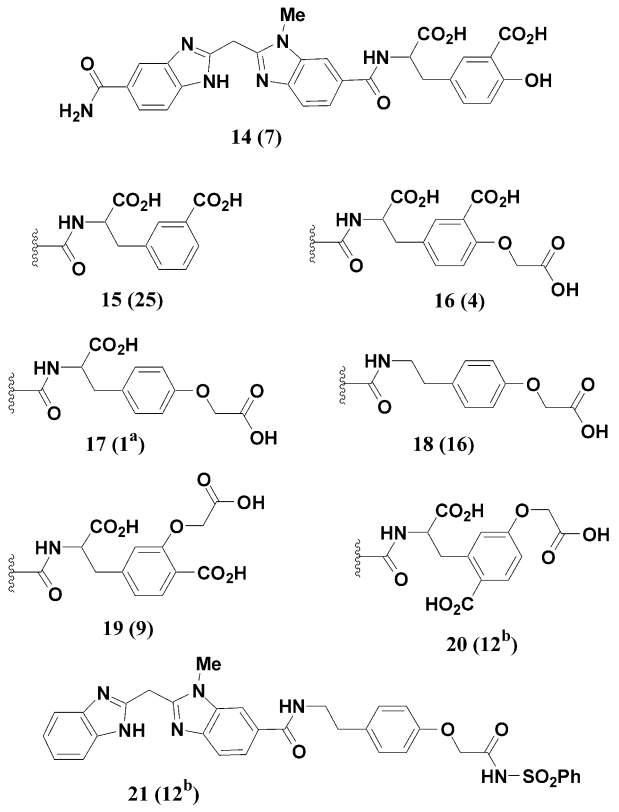


Figure 3. Compounds **14**–**21** and, in parentheses, their IC_{50} (μM) against full-length NS3 in the presence of Zn^{2+}.[7] (a) Same activity against NS3 protease domain (aa 1–183); (b) Activity against NS3 protease domain (aa 1–183).

additional polar functionality to the phenyl ring led to decreased potency (compare **17** with **16**, **19**, and **20**). The inhibitory activity shows little sensitivity to the relative topology of the acetic acid moiety since the *para*-phenoxyacetic acids **16** are only 2- to 3-fold more potent than **19** or **20**. However, the conformational constraint incorporated into tyrosine-*O*-acetic acid **17** suggests that it is not functioning as a direct mimic of **1**, assuming a similar binding mode of the bis-benzimidazole core.[5] It is plausible that **17** identifies a specific binding interaction more closely associated with the acidic moiety of **4** distal to the heterocyclic nucleus.

In order to determine the importance of the α-carboxyl group of the tyrosine-*O*-acetic acid inhibitors, the tyramine analogue **18** was prepared. This compound was an order of magnitude less potent than prototype **17**, a circumstance not altered by increasing the lipophilicity of the carboxyl terminus, as in the acylsulfonamide **21**. These results suggest a substantial contribution by the α-carboxyl group to the HCV NS3 inhibitory activity of this chemotype.

As a complementary extension to this structure–activity survey, constraining the flexibility of the side chain at the α-position was also examined. An aminobenzoic acid template provided a convenient means to explore this concept and the compounds prepared are depicted in Figure 4. From this exercise, it was found that a *meta*-aminobenzoic acid, **24**, was superior to the *para* isomer, **23**. Isophthalate analogues **25** and **26**, in which the additional carboxyl group may function as the α-carboxylate group of **1**, afforded the best potency in this series.

The inhibitors of HCV NS3 protease described in this survey exhibit a relatively flat SAR profile. Although several novel replacements for the phosphonoalanine moiety of **1** were identified that retained reasonable potency, none surpassed the activity of the prototype or its close homologues. Whilst the activity of compound **17**, with an extended tyrosine-*O*-acetic acid side chain, is particularly interesting, it is not clear that this represents the result of a specific binding interaction with the HCV NS3 protein. The results described here are reminiscent of the results reported for a series of inhibitors of HCMV protease designed to take advantage of the concept of Zn^{2+}-stabilization of the inhibitor–protease complex.[11] There appears to be a significant contribution of electrostatic interactions in the vicinity of the active site to the recognition of substrates by HCV NS3 protease.[12–14] The substrate recognition groove and binding pockets of HCV NS3 lack the deep invaginations more typically observed with mammalian serine proteases. As a consequence, a plausible explanation for the results reported herein relies on an initial attractive electrostatic interaction between protease and inhibitor that is subsequently consolidated and stabilized by the zinc ion. The flat and featureless environment of the active site and adjacent substrate recognition sites will likely require a more subtle and well defined stereochemical presentation of a combination of complementary functionality and hydrophobic elements if a more coherent SAR is to be observed and more potent inhibitors are to be identified.

The bisbenzimidazole core acids **27** and **28** were prepared in a similar fashion to the method reported in ref 6. The side chains were coupled to the core acid either as the preformed TMS ester using MSTFA, or as the

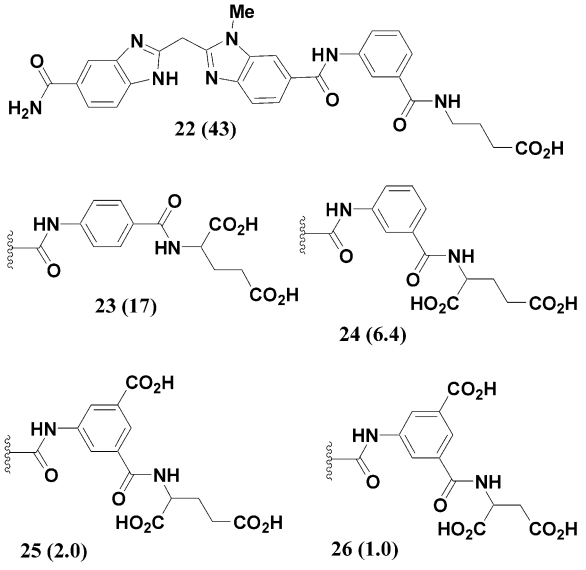
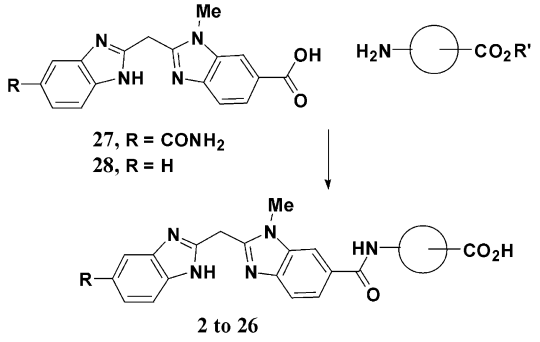


Figure 4. Compounds **22**–**26** and, in parenthesis, their IC_{50} (μM) against NS3 protease domain (aa 1–183) in the presence of Zn^{2+}.[7]

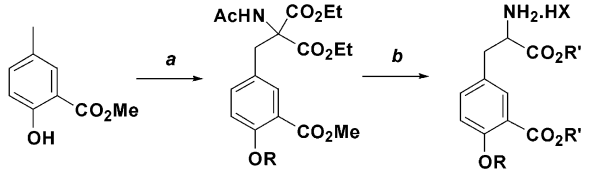
methyl or benzyl ester followed by deprotection under basic hydrolytic or hydrogenolysis conditions (Scheme 1). The use of PyBroP/$^{i}Pr_2NEt$ in DMF appeared to be more generally useful for the coupling than other reagents (e.g., PyBoP/$^{i}Pr_2NEt$, HATU/$^{i}Pr_2NEt$, EDC/HOBT and DPPA/Et_3N). All coupling reactions were performed under a nitrogen atmosphere to minimize air oxidation of the bridgehead methylene carbon. The final products were purified by either reverse-phase preparative HPLC (H_2O/MeOH/0.1% TFA) or flash chromatography on silica gel using EtOAc/^{i}PrOH/aq NH_3 as the eluent.

The side chains of compounds **6**–**13** were prepared from the coupling of the α-benzyl ester of *N*-^{t}Boc-aspartic (or glutamic) acid with methyl 2-(aminosulfonyl)benzoate using EDC/DMAP in CH_2Cl_2, or with iminodiacetic acid dimethyl ester using PyBroP/$^{i}Pr_2NEt$ in DMF.

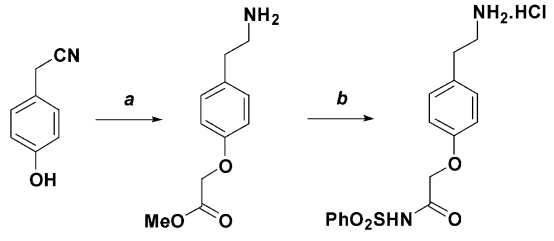
The side chains of compounds **14** and **16** were prepared from methyl 5-methyl salicylate in five steps with the addition of diethyl acetamidomalonate to the intermediate benzylbromide as the key reaction.[15] The side chains of **19** and **20** were prepared analogously using 4-methylsalicyclic acid and ethyl 2-methyl-4-hydroxybenzoate (prepared from the Diel–Alder reaction between 2-(trimethylsilyloxy)-1,3-cyclohexadiene and ethyl tetrolate[16]) as the starting material, respectively (Scheme 2).



Scheme 1. General synthesis of compounds **2**–**26**.



Scheme 2. (a) (i) MeI (for **14**) or methyl bromoacetate (for **16**), K_2CO_3, DMF, rt; (ii) NBS, Bz_2O_2, CCl_4, reflux; (iii) diethyl acetamidomalonate, NaOEt, EtOH, rt; (b) (i) 1 N HCl/H_2O, reflux; (ii) 48% HBr, reflux (for **14**) or concd H_2SO_4, MeOH, reflux (for **16**).



Scheme 3. (a) (i) Methyl bromoacetate, K_2CO_3, DMF, rt; (ii) $NaBH_4$, $CoCl_2$, MeOH/THF, rt (for **18**); (b) (i) Boc_2O, Et_3N, CH_2Cl_2, rt; (ii) NaOH (1 N, aq), MeOH, rt; (iii) $PhSO_2NH_2$, EDC, DMAP, CH_2Cl_2, rt; (iv) 4 N HCl, dioxane, rt (for **21**).

The tyrosine-*O*-acetic acid side chain of compound **17** was prepared by *O*-alkylation of *N*-*t*Boc-tyrosine-α-methyl ester with methyl bromoacetate. The tyramine side chains of **18** and **21** was prepared by *O*-alkylation of 4-hydroxyphenylacetonitrile followed by $CoCl_2$-mediated $NaBH_4$ reduction[17] of the nitrile (Scheme 3).

The side chains of compounds **22**–**26** were prepared from the coupling nitrobenzoic acid or mono-methyl 5-nitroisophthalate to the amino acid methyl ester via the acid chloride ($SOCl_2$), followed by reduction of the nitro group.

Acknowledgements

We thank Dr. Kenneth D. Rice (Axys), and Dr. William Harte and Dr. Michael A. Walker (Bristol-Myers Squibb) for interesting discussions. We also thank Discovery Analytical Sciences (BMS, Wallingford) for collecting some of the ^{1}H NMR spectra.

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were studied separately in more detail but this did not lead to any tractable results.
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