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Design and Synthesis of Ethyl Pyrrolidine-5,5-*trans*-lactams as Inhibitors of Hepatitis C Virus NS3/4A Protease

Martin J. Slater,* David M. Andrews, Graham Baker, Susanne S. Bethell, Seb Carey, Helene Chaignot, Berwyn Clarke, Barry Coomber, Malcolm Ellis, Andrew Good, Norman Gray, George Hardy, Paul Jones, Gail Mills and Ed Robinson

GlaxoSmithKline Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, UK

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Abstract—Using a pyrrolidine-5,5-*trans*-lactam template, we have designed small, neutral, mechanism-based inhibitors of hepatitis C NS3/4A protease. Compound **11a**, with an alpha-ethyl P1 substituent and a Boc-valine substituent at the pyrrolidine nitrogen, has an $IC_{50} = 30\ \mu M$.

An estimated 3% of the global human population is infected by hepatitis C virus (HCV),[1] an infection which often leads to cirrhosis, hepatocellular carcinoma and liver failure in later life.[2] It has been estimated that of those currently infected, 20% and 4% are likely to develop liver cirrhosis and liver cancer, respectively, in the next decade.[3] Current therapies are based upon the use of interferon-α, alone or in combination with ribavirin. Although patients sustained response rates are markedly improved using combination therapies, at least 50% of patients fail to show a sustained response. Additionally, these therapies have the disadvantage of frequent and severe side-effects.[4] The development of new therapies to treat HCV infection effectively is therefore of paramount importance, and is currently an intensive area of research.[5]

HCV is a small enveloped virus, the genome of which is a 9.5 kb single stranded RNA that encodes for a single large polyprotein of 3010–3030 amino acids. This polyprotein is processed by cellular signal peptidases to produce the structural viral proteins (C, E1, E2, p7), whereas viral proteases (NS2, NS3) are responsible for the production of mature non-structural replicative proteins. The multifunctional 70kD NS3 protein is the most extensively studied viral protein.[6] The amino-terminal third of the protein is a trypsin-like serine protease that cleaves the NS3-4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions.

Although NS3 is enzymatically active on its own, NS4A interacts with it to form an integral structural component of the enzyme.[7] The NS3/4A heterodimer modulates protease activity, and is essential for cleavage at the NS3–4A and NS4B-NS5A junctions. It has been reported that when appropriate mutations were made in the NS3 protease region of the HCV genome, the infectivity of these RNAs in chimpanzees was abolished.[8] NS3 protease is thus an essential viral function and an excellent target for the development of novel anti-HCV agents.

A number of peptidic and non-peptidic inhibitors of the serine protease have been reported.[5,9] The former have been mainly competitive inhibitors designed from peptide substrates or cleavage products, whilst the non-peptidic molecules have emerged through random screening and have displayed non-competitive mechanisms of action. As a starting point for the design of inhibitors we sought to take advantage of the pyrrolidine-5,5-*trans*-lactam template which allows access to the S_1, S_1' and S_3–S_4 specificity pockets, and traps the active site serine hydroxyl group by acylation (Fig. 1). These proven templates, developed as thrombin,[10] human neutrophil elastase (HNE)[11] and human cytomegalovirus (HCMV) inhibitors,[12] are active intracellularly, stable in plasma, and orally active in vivo. We

*Corresponding author. Tel.: +44-1438-763416; fax: +44-1438-763620; e-mail: mjs40312@gsk.com

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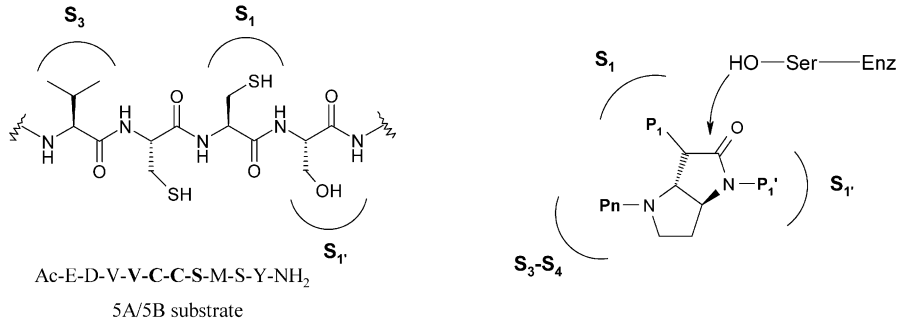


Figure 1. Generic 5,5 pyrrolidine *trans*-lactam template and part of the 5A/5B substrate.

now report the design of such mechanism-based, low molecular weight, non-peptidic 5,5-*trans*-lactam molecules as inhibitors of NS3/4A protease.

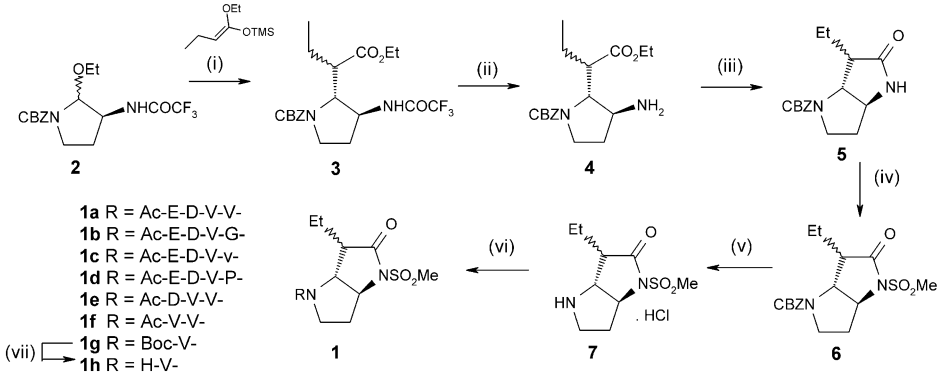
Whilst cysteine is established as the preferred P_1 amino acid for intermolecular cleavage of substrates by NS3, the ethyl group has been shown to be an acceptable inert replacement in substrates and inhibitors,[9] and was chosen as the substituent adjacent to the carbonyl group in the lactam. For the P_1' substituent, we initially selected methanesulphonyl to mimic the serine or alanine commonly found at P_1', and this functionality also serves to activate the lactam moiety. The remaining binding pockets on the non-primed side of the cleavage site (Pn) may then be accessed via substituents at the pyrrolidine nitrogen (Fig. 1). The 5,5-*trans* lactam compound **7** (Scheme 1) thus became our initial target.

The key intermediate **2** was prepared by reported methods[11] and the flexible acyliminium ion route developed by Macdonald and co-workers[11] was adapted to introduce the ethyl side-chain. With **3** in hand, selective hydrolysis of the trifluoroacetamide group (K_2CO_3/EtOH), ring closure with tBuMgCl to form the *trans*-lactam **5**, lactam activation ($MeSO_2Cl$/LiHMDS/THF), and deprotection of the pyrrolidine **6** (H_2/10%Pd,C/HCl/iPrOH) proceeded smoothly to provide the pyrrolidine *trans*-lactam **7** as the hydrochloride salt.[13]

In order to demonstrate the utility of this template against NS3 protease and explore SAR, a large range of carboxylic acids (~200) were coupled to **7** under standard coupling conditions to provide a diverse set of compounds (data not shown). To our surprise, none of these were active up to concentrations of 300 μM, even with long pre-incubation times (up to 18 h). Compounds **5**, **6** and **7** were similarly inactive. At this point we chose to append short peptides to **7** which mimic the P_3–P_6 amino acid residues in the natural substrate. Compound **1a**, containing the natural P_3–P_6 sequence found at the 5A–5B cleavage site (Ac-E-D-V-V), was thus prepared and did indeed inhibit the protease in a time-dependent manner (Table 1).[14] The IC_{50} for **1a** was 19 μM when the compound was pre-incubated with enzyme for 18 h and 69 μM with 2 h pre-incubation.

By making single amino acid replacements and truncating the peptidic portion, we were able to delineate SAR and in Table 1 present key findings. The key role of the L-valine at P_3 is illustrated by the weak activity of the corresponding glycine analogue (**1b**), D-valine analogue (**1c**) and proline analogue (**1d**). A key and surprising observation was that on truncating the side-chain, we observed that a simple Boc-valine substituent (compound **1g**) had similar activity to the multiple-acid-containing-peptide **1a**, and was more active than **1h** (valine).

The data in Table 1 were generated using our initial chromogenic assay to evaluate protease inhibition, employing NS3 protease domain, synthetic 18-mer 4A cofactor, and Ac-E-D-V-V-P-C-pNO$_2$ anilide as substrate.[15] We then moved to a fluorogenic assay system using full-length NS3/4A protein and Abz-E-D-V-V-P-C-S-M-S-(3-NO$_2$)-Y-H as substrate,[15] as this assay has the advantage of delivering linear kinetics and greater



Scheme 1. (i) $BF_3.OEt_2$, DCM; (ii) K_2CO_3, EtOH, 37% from 2; (iii) tBuMgCl, THF; (iv) LiHMDS, $MeSO_2Cl$, THF; (v) H_2, Pd/C, iPrOH, HCl; (vi) HATU, DIPEA, RCO_2H, MeCN or DMF; (vii) HCl, dioxan.

Table 1. NS3/4A protease activities of compounds **1a**–**1h** in the chromogenic assay

Compd	R	% Inhibition at 100 μM 2 h pre-incubation	% Inhibition at 100 μM 18 h pre-incubation
1a	Ac-E-D-V-V-	$IC_{50}=69$ μM	$IC_{50}=19$ μM
1b	Ac-E-D-V-G-	10	24
1c	Ac-E-D-V-(D)V-G-	24	85
1d	Ac-E-D-V-P-	5	11
1e	Ac-D-V-V-	68	94
1f	Ac-V-V-	21	45
1g	Boc-V-	52	$IC_{50}=27$ μM
1h	H-V-	11	41

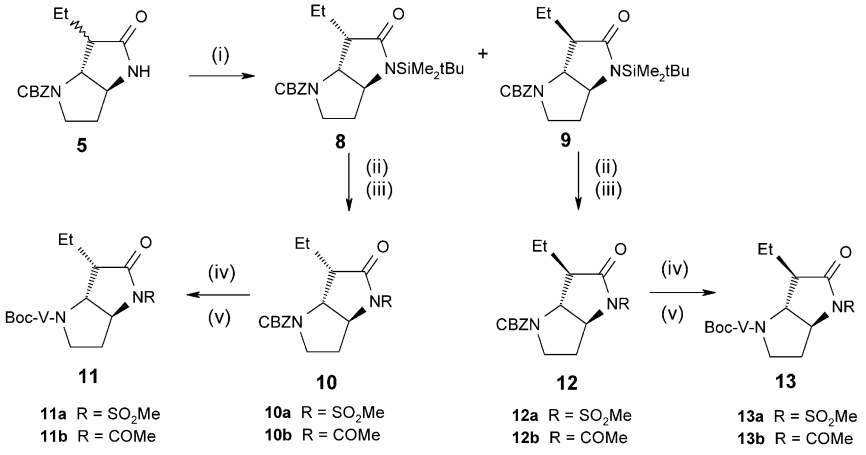
Table 2. NS3/4A protease inhibition

Compd	R	Chromogenic assay		Fluorogenic assay	
		IC_{50}/μM 4 h pre-incubation	IC_{50}/μM 18 h pre-incubation	IC_{50}/μM 4 h pre-incubation	IC_{50}/μM 18 h pre-incubation
1a	SO_2Me	69	19	>400	284
1g	SO_2Me	62	27	182	140
11a	SO_2Me	30	22		
13a	SO_2Me	99	28	341	172
11b	COMe	88	45	193	194
13b	COMe	>400	147	216	447

sensitivity. Cross-checking with this fluorogenic assay showed that the SAR was consistent between assays, although compounds generally displayed higher IC_{50} values in this system (see Table 2).

As **1g** was a 1:1 mixture of *alpha* and *beta* ethyl diastereoisomers, we sought to separate these and determine the preferred stereochemistry of the ethyl substituent for inhibition. In addition to the methanesulphonyl group, we chose also to explore acetyl substitution at the lactam to exemplify a different small activating P_1' substituent (Scheme 2). The isomers of the CBZ-protected pyrrolidine **5** were partially separated by crystallisation from ethyl acetate, which afforded the *beta* isomer (containing 20% of the *alpha* isomer). Silica column chromatography of the mother liqueur (ethyl acetate: cyclohexane, 2:1 as eluant) then provided the *alpha* isomer (containing 25% of the *beta* isomer).[13] A much more efficient separation involved derivatisation of the lactam **5** with the *tert*-butyldimethylsilyl group, as the isomers **8** and **9** could be completely separated by Biotage chromatography (cyclohexane:ethyl acetate, 6:1). Fluoride deprotection using tetrabutyl ammonium fluoride buffered with acetic acid, reaction with methanesulphonyl chloride or acetyl chloride, and exchange of CBZ for Boc-valine was then performed on the individual isomers in the standard manner, to deliver the targets **11a**, **11b**, **13a**, **13b**.

Both *alpha* and *beta* ethyl isomers were active, although the alpha–ethyl compounds (**11a**, **11b**) were found to be more rapid enzyme inhibitors than the *beta*-counterparts (**13a**, **13b**). Methanesulphonyl was a more active lactam substituent than acetyl (Table 2).



Scheme 2. (i) tBDMSCl, LiHMDS, THF; (ii) nBu_4NF, AcOH, THF; (iii) $MeSO_2Cl$ for a, MeCOCl for b, LiHMDS, THF; (iv) H_2, Pd/C, iPrOH, HCl; (v) HATU, DIPEA, Boc-valine, MeCN.

In conclusion, we have designed low molecular weight, neutral inhibitors of the NS3/4A protease using a pyrrolidine *trans*-lactam template. These provide excellent starting points for the design of potential HCV drugs. The further optimisation of these inhibitors will be reported in due course.

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13. The relative stereochemistry is assigned on the basis of coupling constants and NOE experiments in the ^{1}H NMR spectrum, and by comparison with compounds described in references 11 and 12.
14. The detailed kinetics of enzyme inhibition and mechanism of action will be the subject of a separate publication.
15. Chromogenic assay: Enzyme concentration was 0.56 μM, 4A concentration was 0.8 μM, substrate concentration was 1.5mM, substrate $K_m = 1.85$mM. Flourogenic assay: Enzyme concentration was 20 nM, NS3/4A ratio 1:1, substrate concentration was 25 μM, substrate: $K_m = 5$ μM.