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Phosphorous acid analogs of novel P2–P4 macrocycles as inhibitors of HCV–NS3 protease

Marco Pompei [a,*], Maria Emilia Di Francesco [a], Uwe Koch [a], Nigel J. Liverton [b], Vincenzo Summa [a]

[a] IRBM (Merck Research Laboratories), Rome, Italy
[b] Merck Research Laboratories, West Point, PA 19486, United States

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ABSTRACT

HCV–NS3 protease is essential for viral replication and NS3 protease inhibitors have shown proof of concept in clinical trials. Novel P2–P4 macrocycle inhibitors of NS3/4A comprising a P1 C-terminal carboxylic acid have recently been disclosed. A series of analogs, in which the carboxylic residue is replaced by phosphorous acid functionalities were synthesized and found to be inhibitors of the NS3 protease. Among them the methylphosphinate analogue showed nanomolar level of enzyme inhibition and sub-micromolar potency in the replication assay.

Hepatitis C virus (HCV) infection is the major causative agent of chronic liver disease and it is estimated that more than 170 million individuals worldwide are infected with this pathogen.[1] Currently, the only FDA-approved regimen for the treatment of HCV infection is a combination of alpha interferon and ribavirin, a therapy which is poorly tolerated and has a suboptimal response particularly in patients infected with HCV-genotype 1 (gt1), the most prevalent virus genotype.[2] HCV is a small, enveloped, single stranded positive RNA virus belonging to the *Flaviviridae* family.[3] To date, amongst the inhibitors of the HCV–NS3 serine protease so far discovered,[4] the structurally optimized product-derived analogs represent the most promising category.[5]

Clinical proof of concept was achieved with BILN-2061,[6] (Fig. 1) a rapidly reversible inhibitor. The search for NS3 protease inhibitors incorporating new structural motifs was reinforced by discontinuation of clinical evaluation of BILN-2061 due to its cardiac toxicity.[7] This prompted several groups to investigate optimization of both the peptide backbone[8] and the C-terminal P1 carboxylate region.[9] Through this approach, our laboratories identified a novel class of P2–P4 macrocycle analogues as inhibitors of HCV–NS3 protease.[10] Among them, MK-7009 (**1**), a potent inhibitor bearing a cyclopropylacylsulfonamide in P1, showed strong antiviral activity in HCV infected chimpanzees and is currently being evaluated in clinical trials.[11] Previous studies, focused on product based inhibitors, have extensively investigated the impact of different carboxylic acid replacements (e.g., tetrazole, acylcyanamide, acylhydrazine and acylsulfonamide)[22] and led to the discovery of the cyclopropylacylsulfonamide fragment as the preferred P1 carboxylic acid replacement.[12] As part of our strategy to identify novel P2–P4 macrocyclic inhibitors of NS3/4A, we planned to evaluate alternative carboxylic acid bioisosters and focused specifically on phosphonates/phosphinates as potential acid replacements. These moieties have been widely studied as acid bioisosters and have been exploited to develop novel series of biologically active compounds such as enzyme inhibitors,[13] but very few examples are described in the specific case of HCV–NS3 protease.[14] Phosphonic acids, classified as non-planar bioisosteric surrogates of the carboxylic acid function,[15] can be involved both in electrostatic interactions and hydrogen bonds. Therefore, although differences might exist between the two functional groups in terms of key interactions with the enzyme active site,[16] we became interested in exploring the incorporation in our class of P2–P4 macrocycles of acidic phosphorous groups in the P1 position. In this letter we report the synthesis and in vitro evaluation of a series of novel P2–P4 macrocycles bearing a phosphonic or phosphinic moiety as a replacement of the P1 carboxylic acid functionality (Fig. 2).

The synthesis of the P1 phosphorous building blocks **5** and **9** is shown in Scheme 1.

Commercially available diethyl phosphonate **3** was converted in two steps into diethyl-(*N*-benzylidenaminomethyl)-phosphonate (**4**). Reaction with (2*E*)-1,4-dibromobut-2-ene in the presence of

* Corresponding author. Tel.: +39 06 91 09 32 83; fax: +39 06 91 09 36 54.
E-mail address: marco_pompei@merck.com (M. Pompei).

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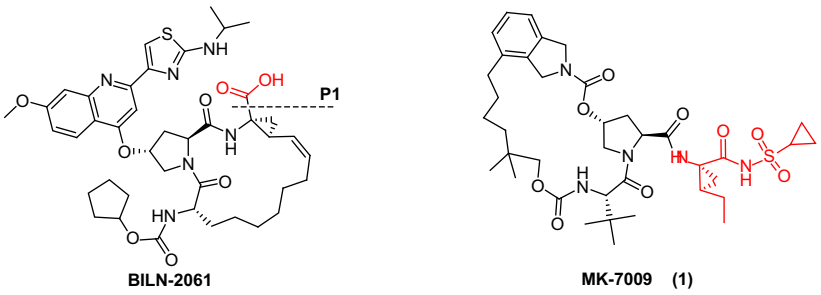


Figure 1. NS3/4A protease inhibitors.

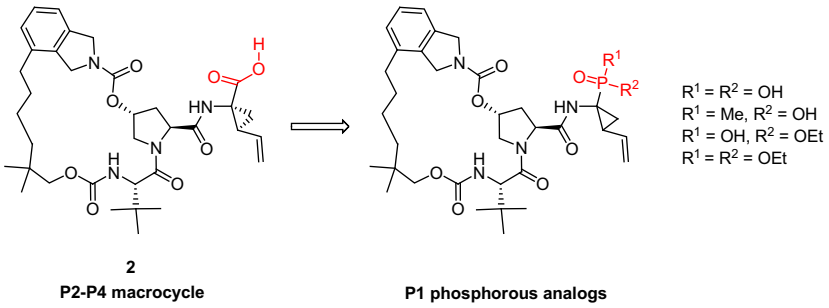
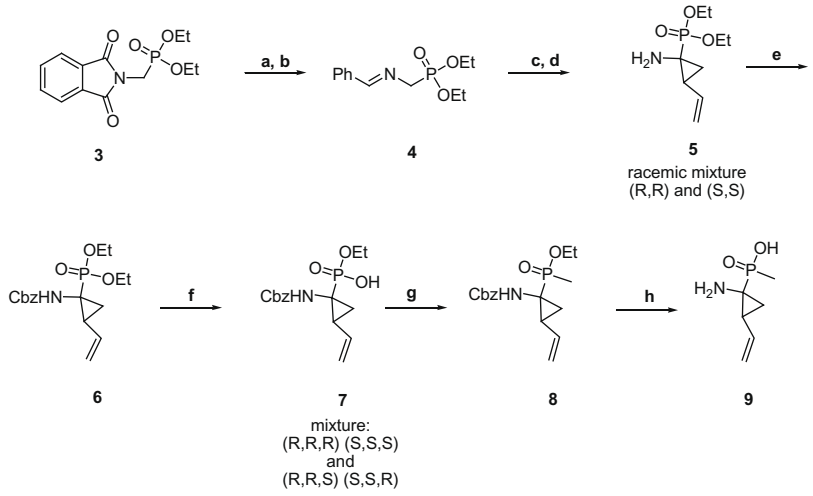


Figure 2. Targeted phosphorous acid analogues in P1.



Scheme 1. Preparation of intermediates **5** and **9**. Reagents and conditions: (a) N_2H_4, EtOH, rt; (b) PhCHO, toluene, reflux (38% over two steps); (c) *trans*-1,4-dibromo-butene, CsOH, rt, DCM; (d) HCl $_{(aq)}$, rt; (17% over two steps); (e) CbzCl, Na_2CO_3, DCM, rt (37%); (f) NaI, Py, 110 °C (90%); (g) $(COCl)_2$, DMF, 0 °C; then MeLi, −78 °C, DCM (38%); (h) TMSI, DCM, 0 °C (50%).

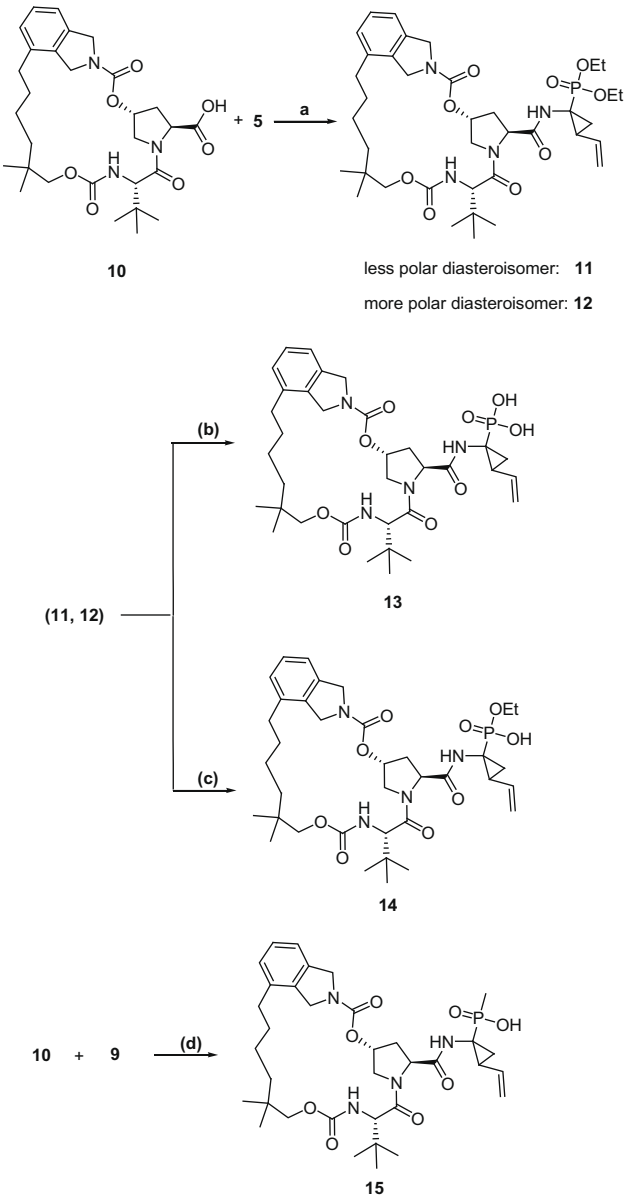
cesium hydroxide followed by hydrolysis in acidic conditions afforded racemic amine **5**.[17] The methyl-phosphinic acid **9** was then obtained as a mixture of diastereoisomers in four steps from intermediate **5**. In particular, the Cbz protection of the amino group afforded species **6** that was in turn selectively mono-hydrolyzed with NaI in pyridine to the diastereomeric mixture of mono-ethyl phosphonate esters **7**. Reaction with oxalyl chloride and catalytic DMF in dichloromethane afforded the corresponding phosphonochloridoate which was then reacted at −78 °C with methyllithium to give, after complete deprotection, the required methyl-phosphinic acid **9**.

The synthesis of the target products is outlined in Scheme 2. The key macrocyclic acid **10** was prepared in overall good yield according to the published procedure.[18,11]

Finally, the targeted compounds **11**, **12**, **13** and **14** were obtained assembling the P2 and P1 fragments previously prepared as outlined in Scheme 2.

Coupling reaction of **10** with **5** in presence of HATU gave the diasteroisomeric products **11** and **12** that were separated by preparative HPLC. Mono and di ester hydrolysis were accomplished by appropriate choice of conditions. Thus, reaction of the diasteroisomeric mixture of **11** and **12** with TMSI afforded the phosphonic acids **13** in moderate yield, while mono esters **14** were obtained by reaction with sodium iodide in pyridine. The methyl phosphinic species **15** were finally obtained by coupling reaction of **10** with **9** in presence of α-ethylchloroformiate.

The compounds synthesized were assessed for enzymatic potency against genotype 1b NS3/4A[19] and in a subgenomic

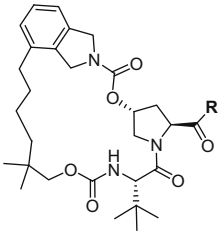


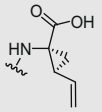
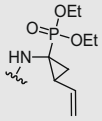
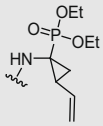
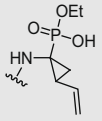
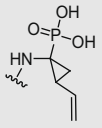
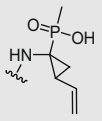
Scheme 2. Preparation of intermediates **11**–**15**. Reagents and conditions: (a) HATU, $^{i}Pr_2EtN$, DCM, rt (10%); (b) TMSI, ACN, rt (15%); (c) NaI, Py, rt (12%); (d) EtOC(O)Cl, TEA, DCM, rt (11%).

replication assay in Huh-7 cells (replicon).[20] In this assay, potency was measured both in the presence of 10% Fetal Calf Serum (FCS) and of 50% Normal Human Serum (NHS), the latter to assess the impact of protein binding.

The resulting data are reported in Table 1, including the carboxylic acid **2** as comparator to the above reported phosphorous inhibitors. Compounds **11** and **12** were tested as single stereoisomers and compounds **13**, **14** and **15** as mixture of diasteroisomers.[21] The two compounds **11** and **12**, with a neutral ethyl-phosphonate group, presented modest levels of intrinsic and cellular potency (K_i = 2.9 μM and EC_{50} 10% FCS = 20 μM) independently of the stereochemistry of the ethenylcyclopropyl group. Interestingly the enzymatic potency of **11** and **12** was comparable to the charged carboxylic acid **2**, and suggested that the phosphonic replacement was tolerated within the active site of NS3 protease. The introduction of a acidic phosphorous moiety in compounds **13** and **14** provided a dramatic increase in activity (K_i = 0.016 and 0.070 μM, respectively) supporting the hypothesis that a charged acid group

Table 1
HCV–NS3 protease inhibition constants and EC_{50} values



Compd	R	K_i[a] (μM) 1b	EC_{50}[b] (μM) 10% FCS/50% NHS	pK_a[c]
2[11]		2.8	–	3.4
11[d]		2.9	20/15	–
12[d]		2.9	20/20	–
13[e]		0.016	5.0/5.0	2.3
14[e]		0.070	0.66/2.4	4.9
15[e]		0.0024	0.50/0.25	3.1

[a] Inhibition of the full-length HCV–NS3/4A protease measured by the inhibition constants (K_i values).
[b] Inhibition of HCV replication in Huh-7-cells measured by 50% effective concentration (EC_{50}) in the presence of 10% Fetal Calf Serum (FCS) or 50% Normal Human Serum (NHS). Results are the mean of at least three independent experiments. SD was ±8% of the value. For details see Refs. 19 and 20.
[c] pK_a calculated using ACDLabs 8.0.
[d] Single stereoisomer.
[e] Mixture of diasteroisomers.

was required for optimal potency. For these compounds the enzyme potency was paralleled by improved cellular activity, more significant for analogue **14**, which showed sub-micromolar potency in low serum conditions (EC_{50} 10% FCS = 0.66 μM). In high serum conditions **14** showed more than threefold shift in replicon potency, with an EC_{50} 50% NHS = 2.4 μM, hinting at the possibility of significant protein binding. The methyl-phosphinate analogues **15** were very potent in the enzyme inhibition assay (K_i = 0.002 μM) with more that 1000-fold increase in activity compared to analogues **12** and **13** and no shift between FCS and NHS (EC_{50} 10% FCS/50% NHS = 0.50/0.25 μM). Further evidence highlighting the potential role of a phosphorous acidic moiety within the enzyme active site can be gained from comparison of the inhibition potency of the analogues **13**, **14** and **15** in relation with their calculated values of pK_a. Specifically, compounds **14**, the least potent among these phosphorous analogues, have a calculated pK_a value of 4.9.

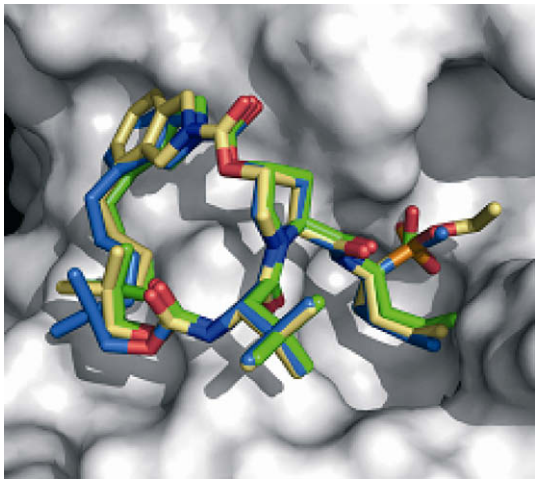


Figure 3. Compounds **13**, **15** and **2** docked in the protease binding site, derived from full-length NS3 protein. Colour code: compound **13** (yellow); compound **15** (blue); compound **2** (green). Oxygen atoms (red), P atoms (orange).

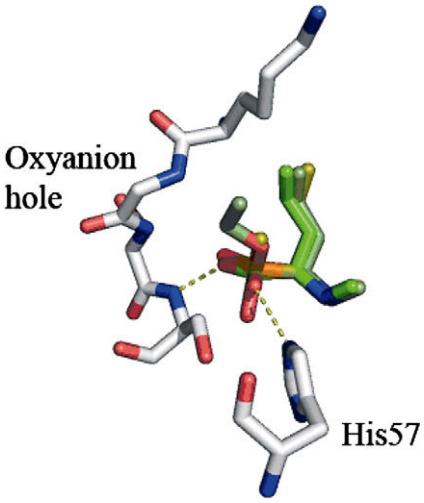


Figure 4. Detail of the P1 region of the P2–P4 phosphorous macrocycles. Superposition of compounds **13**, **15** and **2** and hydrogen bonds in the oxyanion hole and with the catalytic His57.

Accordingly the significant boost in potency observed for **13** and **15** in comparison to **14** might be in part related to the increased acidity of the P1 phosphorous replacement (pK_a = 2.3 and 3.1, respectively). Interestingly, these values are also the closest within the series to those of the carboxylic acid **2** (pK_a = 3.4). These observations are in agreement with previous studies which highlighted the importance of an acidic group in P1 with a value of pK_a close to that of the carboxylic function.[22]

Recent X-ray crystal structures of product inhibitors with a P1 terminal carboxylic acid[23] and docking studies of P1 acylsulfonamide inhibitors[22] bound to the HCV–NS3/4A complex have elucidated the interactions between the protease domain and these acidic functions and given some insight into their mechanism of action. Our additional interest in this potent class of novel phosphorous inhibitors is given by the alkyl-group present in the phosphonate moiety which might offer a handle towards further elaboration to optimize interactions within the active site of HCV–NS3 protease and achieve fine tuning of the physicochemical properties. In order to further strengthen our hypothesis, the single stereoisomers of **13** and **15** with the same relative configuration at the cyclopropryl junction of the analogue **2**, have been modeled into the active site of the HCV–NS3 protease (Fig. 3).

The superposition shows that the phosphonate moiety is capable of forming the typical hydrogen bond in the oxyanion cavity and to the catalytic His57 (Fig. 4). Its binding mode is similar to that of the product inhibitor carboxylate **2**. Due to its steric requirements and not being good hydrogen bond acceptor the methyl group of compound **15** is oriented towards the open region of the catalytic site.

This atomic view of the binding mode of our P1 phosphorous inhibitors, combined with the observed inhibitory potency, highlights the overall good biososteric profile of the P1 acidic phosphorous framework. Moreover, the promising orientation of the methyl group of compound **15** towards the open region of the catalytic site supports the exploitation of further modifications of the phosphinate P1 fragment to gain in additional interactions within the NS3 protease.

In summary, this work identified a novel class of P2–P4 macrocyclic inhibitors of HCV–NS3 protease bearing phosphorous acid groups in P1. The optimal compound showed nanomolar enzyme inhibition and sub-micromolar potency in the cell based assay. The most active analogues were the methyl-phosphinates **15** characterized by a calculated pK_a value close to that of the parent carboxylic acid **2**. With the support of the modeling studies here presented, further work is on-going to explore additional elaboration of the phosphinate moiety in combination with structural modification within the macrocycle. Results will be reported in due course.

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