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Novel P2–P4 macrocyclic inhibitors of HCV NS3/4A protease by P3 succinamide fragment depeptidization strategy

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ABSTRACT

Hepatitis C represents a serious worldwide health-care problem. Recently, we have disclosed a novel class of P2–P4 macrocyclic inhibitors of NS3/4A protease containing a carbamate functionality as capping group at the P3 N-terminus. Herein we report our work aimed at further depeptidizing the P3 region by replacement of the urethane function with a succinamide motif. This peptidomimetic approach has led to the discovery of novel P2–P4 macrocyclic inhibitors of HCV NS3/4A protease with sub-nanomolar enzyme affinities. In addition to being potent inhibitors of HCV subgenomic replication, optimized analogues within this series have also presented attractive PK properties and showed promising liver levels in rat following oral administration.

Hepatitis C virus (HCV) infects an estimated 300 million people world-wide. This infection is the major causative agent of end-stage liver disease and failure.[1] Current therapy involves a combination of pegylated interferon agents (pegIFN)-α with ribavirin, a broad spectrum nucleoside analogue antiviral.[2]

Since NS3/4A has been identified to be essential in HCV replication,[3] several research groups have actively worked toward the identification of compounds targeted to inhibit this chymotrypsin-like serine protease. Both noncovalent inhibitors containing a C-terminal carboxylic acid (e.g., BILN-2061) or an acid bioisoster (e.g., ITMN-191 and TMC-435) and covalent reversible serine-traps (e.g., VX-950 and SCH-503034) have reached advanced phase clinical studies and validated HCV NS3/4A as target for anti-hepatitis C therapeutics.[4]

Novel P2–P4 macrocyclic inhibitors of HCV protease have been reported by our organization to be potent inhibitors of this enzyme[5,6] (Fig. 1), and MK-7009, belonging to the same structural class, is currently in clinical investigation.[7]

As part of a broader discovery program, we have investigated a number of modifications of the capping group at the P3 N-terminus in order to identify valuable replacements of the urethane function and further reduce the peptide character of our proprietary macrocycles.[8]

Working in that direction, we report herein the results of our initial studies aimed at the evaluation of the succinamide fragment as a novel replacement of the P3 capping group (Fig. 2). Amongst peptidomimetic approaches, the succinic acid fragment depeptidization has been extensively reported in various unrelated examples of protease inhibitors as an effective tool to combine potency with suitable PK properties.[9,10] In the specific case of NS3/4A inhibitors, these macrocyclic systems interact within the substrate binding site of the protease through a combination of hydrogen bonds and side-chain hydrophobic interactions. Both crystal structures of natural cleavage products bound to the enzyme,[11] and molecular modeling studies of the P2–P4 macrocyclic inhibitors docked within the substrate binding site,[5a] suggest that the NH and carbonyl groups of the P3 residue are engaged in hydrogen bonds with the carbonyl and NH groups of the backbone aminoacid Ala157. In light of these data, we decided to investigate the tolerability in our class of inhibitors for the replacement of the P3-carbamate capping group with the succinic acid fragment. Despite the elimination of the key P3–NH hydrogen bond to the Ala157 residue, we were nevertheless intrigued by the possibility of preserving the overall pattern of interactions of these P3 succinamide analogues within the protease. To support our hypotheses, we specifically modeled carbamate **2** in the context of the full

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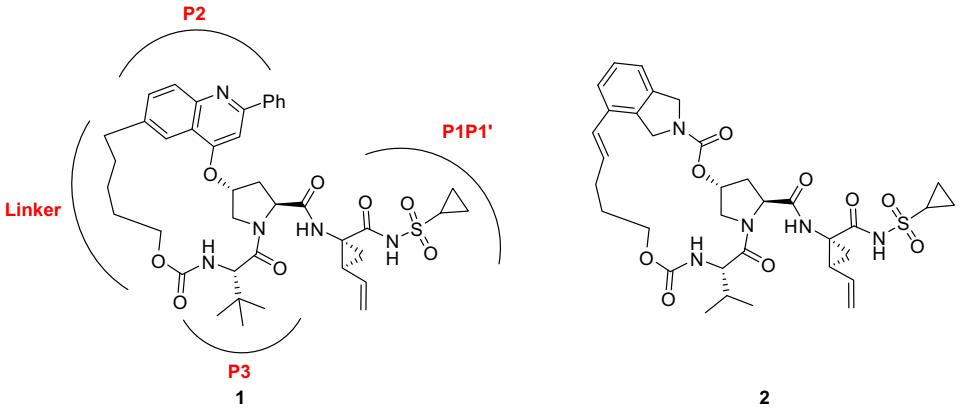


Figure 1. NS3/4A protease inhibitors.

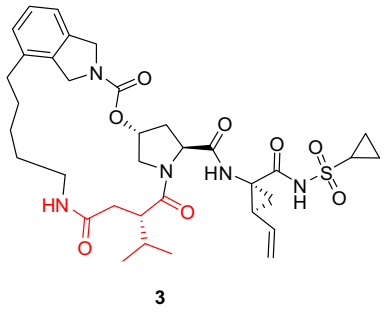


Figure 2. Example of targeted P3-succinamide analogues: compound **3**.

length NS3/4A protease and superimposed it to the succinamide derivative **3** (Figs. 2 and 3).

Pleasingly, the modeling study suggested that the general conformation of the proposed succinamide macrocyclic inhibitor remained mostly unchanged despite the replacement of the P3 aminoacid NH with a methylene group. The introduction of the succinamide moiety was tolerated and allowing a global interaction within the substrate-binding site of the protease comparable to that predicted for the inhibitor **2**.

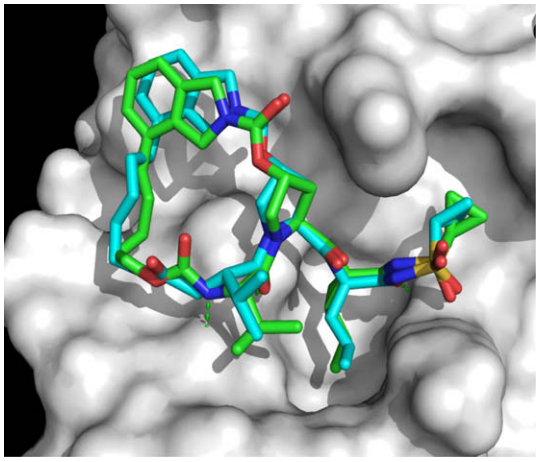


Figure 3. Superposition of compound **2** (green) with the succinamide analogue **3** (cyan) into the substrate binding site of the genotype 1b NS3/4A crystal structure. Both models were obtained via conformational analysis with Batchmin V9.4 using the OPLS2005 force field. Inhibitors were minimized and docked into the active site of the genotype **1b** NS3/4A crystal structure. Potential hydrogen bonds are shown by broken lines.

These results encouraged the evaluation of a preliminary number of derivatives. Initially, the exploration was focused on analogues bearing the isoindoline moiety in P2 and introducing a limited number of structural variations. In particular, the succinamide fragments, 2-isopropyl and 2-cyclohexyl, were selected on the basis of previously developed SAR, which had identified valine and cyclohexylglycine (c-Hex) residues amongst the preferred P3.[12]

Moreover, we intended to investigate if the effect of the substitution at the succinamide nitrogen could be exploited to engage the enzyme in further hydrophobic interactions with possibly positive effects on potency and on pharmacokinetic properties of the corresponding analogues. The substitution on the N-amide atom was initially evaluated by a direct comparison between the N-^{i}Pr and N–H compounds. The choice of this small aliphatic substituent derived both from previously developed SAR on acyclic systems[13] as well as from studies on related P2–P4 macrocyclic inhibitors.[14]

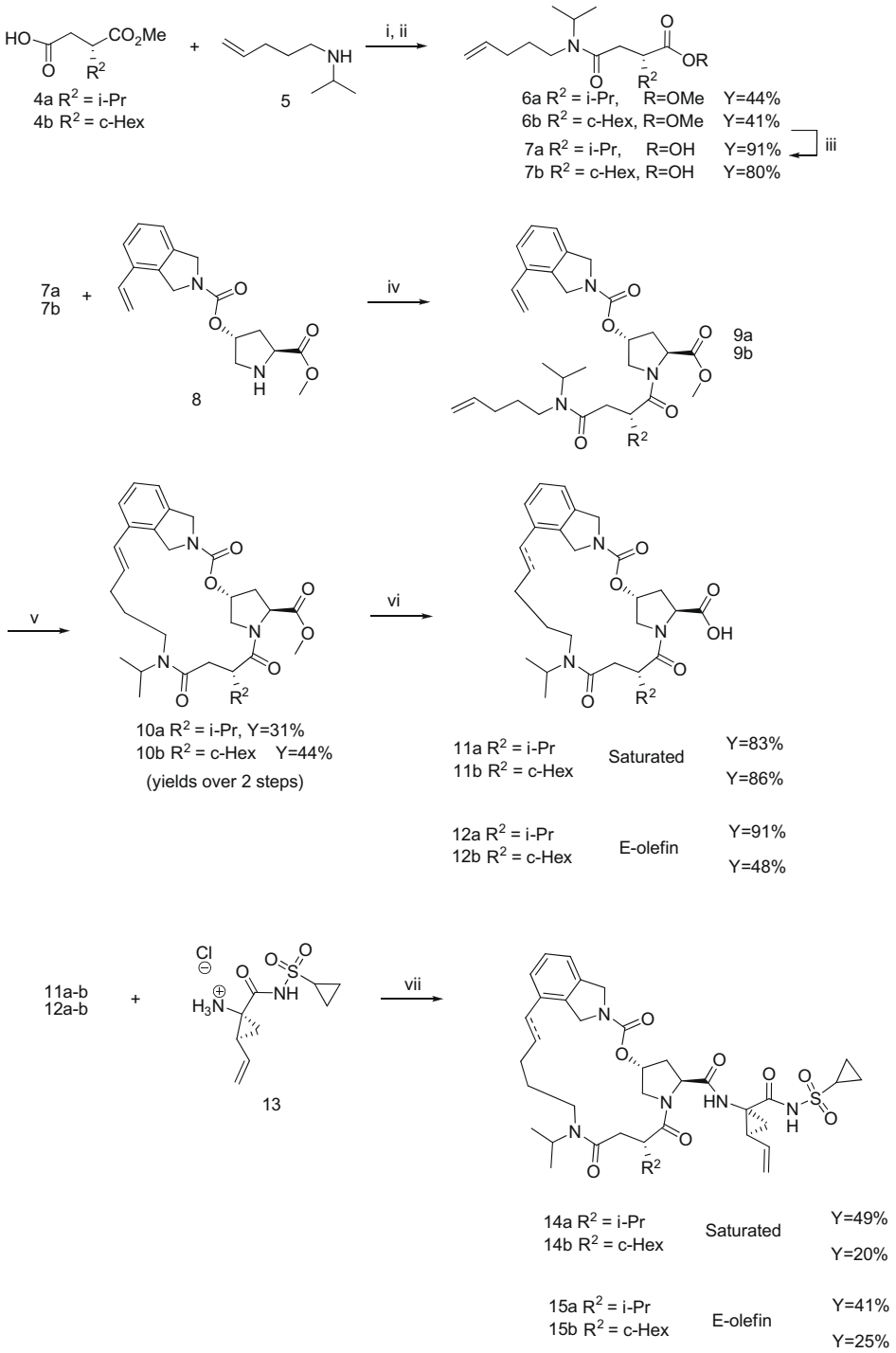
The synthetic approach toward the proposed targets relied on ring closing metathesis (RCM) to perform the key macrocyclization step and is illustrated in Scheme 1, starting with the preparation of the N-^{i}Pr substituted analogues **14a–b** and **15a–b.**

The commercially available succinic acids **4a** and **4b** were first converted into the corresponding acyl chlorides and then coupled with the amine **5** in presence of triethylamine. Hydrolysis of the methyl esters and coupling of the corresponding carboxylic acids with the proline derivative **8**[14] gave intermediates **9a** and **9b**; precursors to the key macrocyclization step**.**

The latter were then progressed to **10a** and **10b** via a microwave assisted RCM reaction with Zhan-1 catalyst.[15] In both cases only the *E*-isomers were isolated after chromatography, and the trans geometry was assessed measuring the vicinal coupling constant of the two protons on the double bond (15–17 Hz). The synthesis was then completed by hydrogenation, hydrolysis of the proline methyl ester and final amide coupling with **13**[16] in presence of TBTU to obtain the saturated compounds **14a–b** after purification by reverse-phase high performance liquid chromatography (RP-HPLC). The corresponding unsaturated analogues **15a–b** were obtained in similar way from the *E*-macrocycles **12a–b**.[17]

Turning next the attention toward the synthesis of the N-unsubstituted succinamide analogues, and according to the previous synthetic approach, amine **17**[18] was at first coupled with the succinic acid **4b** via the corresponding acyl chloride. Surprisingly, a 3:1 inseparable mixture of the positional isomers **18** and **19** was obtained in this case (Scheme 2).

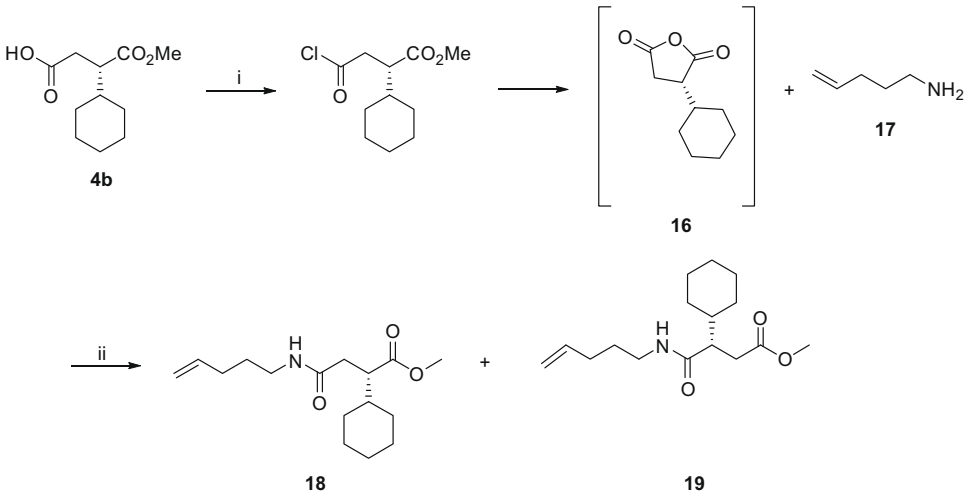
Reasoning the high reactivity of the intermediate acyl chloride, with the plausible involvement of anhydride **16,** could account for



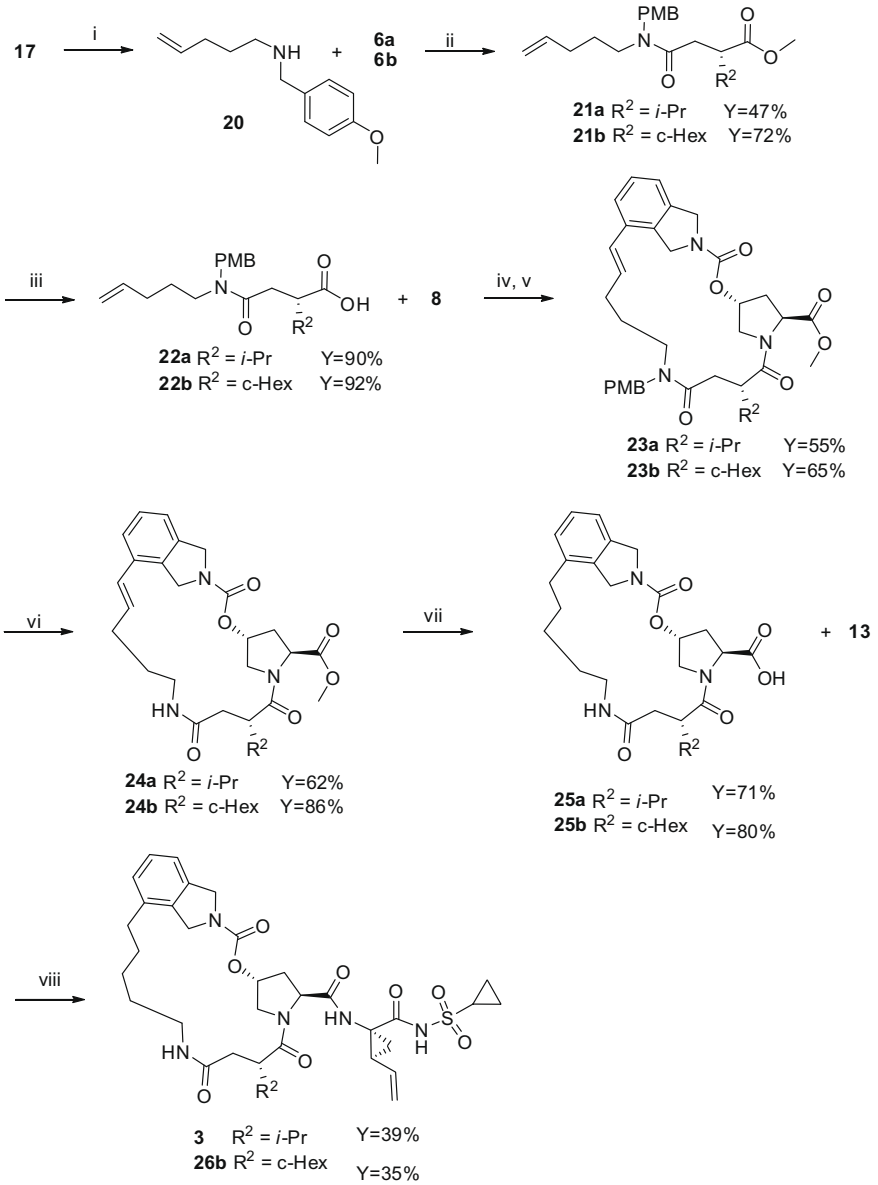
Scheme 1. Synthesis of analogues **14a–b** and **15a–b**. Reagents and conditions: (i) oxalyl chloride, DMF (cat.), DCM, rt; (ii) TEA, DCM, rt; (iii) LiOH, dioxane/water 1:1, 50 °C; (iv) BOP, *N*-methyl morpholine, DCM, rt; (v) Zhan-1 catalyst, DCM, μW irradiation, 100 °C, 10 min; (vi) unsaturated analogs: LiOH, THF/dioxane 1:1, rt; saturated analogs: Pd(C) 10%, H_2, MeOH, then LiOH THF/dioxane 1:1, rt; (vii) TBTU, DIPEA, DCM, RP-HPLC purification.

the observed outcome, the initial exploration of alternative and milder coupling conditions was set up with the aim of improving the selectivity in favor of the required regioisomer **18**. Unfortunately, in all the cases evaluated (e.g., 2-bromo-1-ethylpyridinium tetrafluoroborate, HOBT in presence of diethylamine[19] or EDC, HOBT and triethylamine[20]) the coupling reactions gave inseparable mixtures of **18** and **19,** with no practical improvements in the isomeric ratio. In order to circumvent these problems, a different disconnection approach was also screened and based on the amide coupling step between **8** and (2*S*)-4-*tert*-butoxy-2-cyclohexyl-4-oxobutanoic acid. Unfortunately, complex mixtures of positional isomers were observed in this case too, independently of the coupling reagent system employed (data not reported).

In contrast with these results, the secondary amine **5**, as previously shown, discriminating between the two carbonyl groups of anhydride **16**, most probably on steric grounds**,** gave a straightforward and completely regioselective synthesis of the required succinamides **6a–b**. In light of these observations, the solution to the



Scheme 2. Preparation intermediates **18** and **19**. Reagents and conditions: (i) oxalyl chloride, DMF (cat.), DCM, rt; (ii) TEA, DCM, rt.



Scheme 3. Synthesis analogues **3** and **26b**. Reagents and conditions: (i) *p*-anisaldehyde, $NaBH_4$, DCM, rt; (ii) HOBT, EDC, TEA, DCM, rt; (iii) LiOH, dioxane/water 1:1, 50 °C; (iv) BOP, *N*-methyl morpholine, DCM, rt; (v) Zhan-1 catalyst, DCM, μW irradiation, 100 °C, 10 min; (vi) TFA 0.1 N, rt; (vii) LiOH, dioxane/water 1:1, rt, then Pd(C) 10%, H_2, MeOH, rt; (viii) TBTU, DIPEA, DCM, RP-HPLC purification.

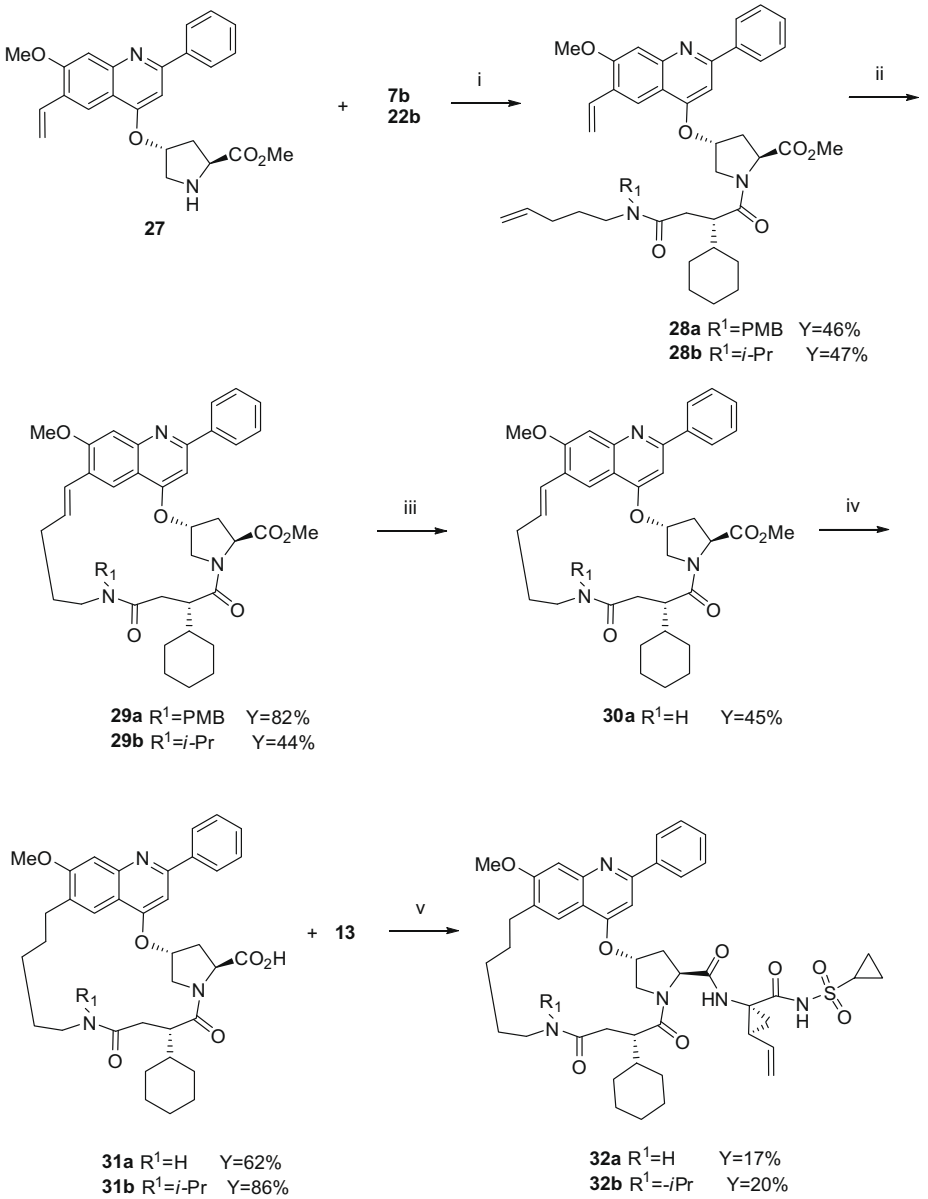
regioselectivity issue was finally identified in the installation of a transient N-protecting group on the amine fragment, exploiting the potential steric bias observed. The choice fell on the N-4-methoxybenzyl group (PMB) in view of its facile cleavage in mild acidic conditions.[21] Indeed, by modifying the synthetic sequence as depicted in Scheme 3, the required N-substituted succinamide building blocks **22a–b** were generated from amine **20**[22] in high yields and with complete regioselectivity, as confirmed by NMR structural elucidation.[23]

Amides **22a–b** were then progressed to the macrocyclic succinamide derivatives **23a–b** according to the previously described synthetic route. Deprotection of the N-PMB group to give intermediates **24a–b** was accomplished with TFA at room temperature. Finally, in a similar fashion to the procedure already described in Scheme 1, the targeted compounds **3** and **26b** were obtained in overall acceptable yields after RP-HPLC purification.

Having identified an efficient synthetic route enabling access to both the N-substituted and the N-unsubstituted succinamide analogues, the investigation was extended to evaluate structurally distinct P2-heterocyclic cores such as the 7-methoxy-2-phenylquinoline, also previously employed by optimization studies within related series of macrocyclic inhibitors.[5] The synthesis of analogues **32a** and **32b** are described in Scheme 4.

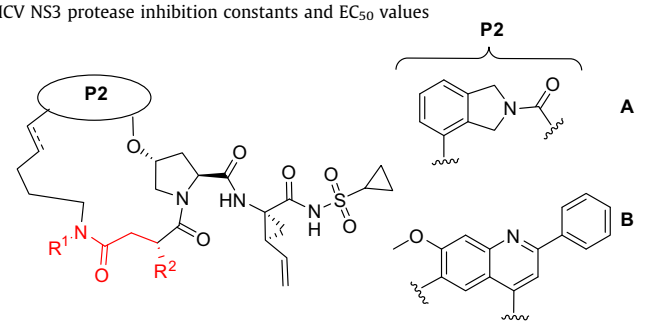
All the succinamide analogues prepared were tested against full-length genotype 1b NS3/4A[24] and in subgenomic replication assay against genotype 1b in Huh-7 cells with a luciferase reporter read-out.[25] The corresponding data are showed in Table 1.

Replacement of the P3-carbamate with the succinamide backbone produced analogues with intrinsic potency in the low-nanomolar range. In particular, this insertion proved partially detrimental, as apparent from the direct comparison amongst **2**[26] (K_i = 0.06 nM) and **3** (K_i = 1.4 nM). In the unsubstituted series the introduction of the cyclohexyl-succinamide fragment as in **26b** resulted in a marginal improvement of the observed K_i value (K_i = 0.76 nM) but with a rather significant boost in the replicon potency (EC_{50} = 12 nM). The presence of the ^{i}Pr group on the succinamide nitrogen, as in analogues **14a** and **14b**, although not associated with a further boost in enzyme affinity (K_i = 0.72 nM and



Scheme 4. Synthesis of analogues **32a**–**b**. Reagents and conditions: (i) BOP, *N*-methyl morpholine, DCM, rt; (ii) Zhan-1 catalyst, DCM, μW irradiation, 100 °C 10 min; (iii) TFA 0.1 N, rt; (iv) Pd(C) 10%, H_2, MeOH, then LiOH THF/dioxane 1:1, rt; (v) TBTU, DIPEA, DCM, RP-HPLC purification.

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



Compd	P2	Linker[a]	R^1	R^2	K_i (nM)[b] **1b**	EC_{50} (nM)[c] 10% FCS
2[d, 26]	A	db	—	^{i}Pr	0.06	12
3	A	Satd	H	^{i}Pr	1.4	100
26b	A	Satd	H	c-Hex	0.76	12
14a	A	Satd	^{i}Pr	^{i}Pr	0.72	35
14b	A	Satd	^{i}Pr	c-Hex	0.61	11
15a	A	db	^{i}Pr	^{i}Pr	16	284
15b-	A	db	^{i}Pr	c-Hex	14	276
32a	B	Satd	H	c-Hex	0.56	7
32b	B	Satd	^{i}Pr	c-Hex	0.60	37

[a] Satd = saturated analogs, db = *E* double bond.
[b] Inhibition of the full-length HCV NS3/4A protease measured by the inhibition constants (K_i values).
[c] 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 ±10% of the value.
[d] Compound **2** is the carbamate-based inhibitor used as comparator within the succinamide series.

0.61 nM, respectively), maintained the good levels of replicon potency observed in 10% FCS (EC_{50} = 35 nM and 11 nM, respectively).

The features observed for these analogues seemed to be quite severely affected by the introduction of an *E*-double bond within the linker chain, as shown by derivatives **15a–b**. These compounds lost up to ca. 10-fold in enzyme potency and showed unfavorable replicon profile in comparison with the saturated analogues **14a–b** and **3–26b**. These findings were rather unexpected, especially given that in related macrocyclic series the presence of an *E*-double bond within the linker was generally well tolerated.[5]

The introduction of the 7-methoxy-2-phenylquinoline as P2 residue resulted in compounds **32a–b** with enzyme affinity in the sub-nanomolar range and equipotent to **14b** and **26b** (K_i = 0.56 nM and K_i = 0.60 nM). In terms of replicon potency, the methoxy-phenyl-quinoline core in P2 also proved to be well tolerated as shown by analogue **32a** with comparable level of activity to the reference compound **2** in the replicon assay (EC_{50} = 7 nM). In view of these results, compounds **32b**, **14a**, **14b**, **26b** and **32a**, were selected to be further profiled in rat, with the initial aim to evaluate plasma and liver levels following oral administration. The compounds were dosed orally in Wistar rats at 4 mg/kg and the data are reported in Table 2. Analogues **14a** and **14b** showed comparable plasma exposures (AUC = 0.12 μM h and AUC = 0.22 μM h, respectively) in combination with encouraging levels of liver concentration at 4 h, in both cases in the micromolar range (2.2 μM and 3 μM, respectively). However, the favorable profile observed for these first two analogues was not paralleled by the N-unsubstituted compound **26b**.

In fact, the latter showed a reduced plasma exposure and a significant drop in liver levels (AUC = 0.09 μM h and [liver] = 0.38 μM).

A similar trend was also observed for the quinoline derivatives **32a** and **32b**. While **32a**, the N-unsubstituted analogue, had undetectable plasma levels and minimal liver concentration, **32b**, the corresponding N-^{i}Pr substituted compound, showed a much more favorable profile. In particular, the plasma exposure was remarkably improved compared to all the other analogues in the series (C_{max} = 0.38 μM, AUC = 1.1 μM h), with a significant increase also in liver levels (9.3 μM). Despite the variability of the in vivo data within this series, compound **32b** showed a very promising profile and the need for further investigation of closely related analogues.

Table 2
Rat PK data (P.O., 4mpk) for selected compounds

Compd[a]	C_{max} (μM)	Plasma $AUC_{0-\infty}$ (μM h)	4 h liver concn (μM)
14a	0.08 ± 0.01	0.12 ± 0.01	2.2 ± 0.1
14b	0.09 ± 0.02	0.22 ± 0.05[b]	3 ± 1
26b	0.05 ± 0.02	0.09 ± 0.05	0.38 ± 0.08
32a	BLQ	—	0.10 ± 0.04
32b	0.38 ± 0.01	1.1 ± 0.1	9.3 ± 0.1

[a] Compounds dosed at 4 mg/kg P.O. in PEG 400; data are means of three animals.
[b] 0–4 h.

In summary, this study has explored the possibility of replacing the P3 carbamate with a succinamide motif in the context of P2–P4 macrocyclic inhibitors of HCV protease. The initial evaluation of this depeptidization strategy, generated analogues such as **14b** and **26b** with sub-nanomolar enzyme potencies and good cell based activities (K_i = 0.61 nM and K_i = 0.76 nM, EC_{50} = 11 nM and EC_{50} = 12 nM 10% FCS, respectively), suggesting that the succinamide motif might be considered a suitable peptidomimetic approach also in the case of P2–P4 inhibitors. These data confirmed that the introduction of substitution on the succinamide nitrogen did not prevent the interaction of this class of inhibitors within the substrate binding site of the enzyme. Moreover, this substitution showed favorable effects on plasma exposure and liver levels of the corresponding derivatives as consequence of a possible modulation of their physicochemical properties. Extension of the initial investigation to other P2 heterocyclic moieties resulted in the identification of the 7-methoxy-2-phenylquinoline analogue **32b**, a sub-nanomolar enzyme inhibitor characterized by encouraging levels of replicon potency and remarkable liver levels when dosed orally in rat, a key feature for any potential treatment for HCV (K_i = 0.60 nM, EC_{50} = 37 nM; liver concentration at 4 h from oral dosing at 4 mg/kg = 9.3 μM).

In view of these encouraging initial data, further preclinical exploration is underway within this series and aimed to expand the combinations of P2 heterocyclic cores with substituents on the succinamide nitrogen optimized at the S4 pocket of the protease.

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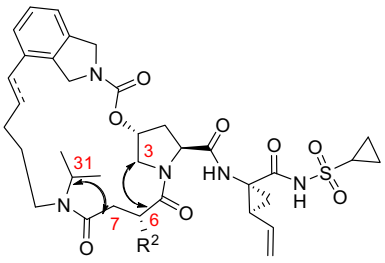
17. The structure of final compounds was assessed by ^{1}H–^{1}H ROESY experiment (key ROE contacts: H31/H7,H7′ and H6/H3).



Representative analytical data for compound **15a**: ^{1}H NMR (600 MHz, DMSO-d_6, T = 300 K) δ 10.67 (s, 1H), 9.26 (s, 1H), 7.31 (d, J = 7.6 Hz, 1H), 7.26 (t, J = 7.6 Hz, 1H), 7.18 (d, J = 7.6 Hz, 1H), 6.59 (d, J = 16.4 Hz, 1H), 6.25 (dt, J_1 = 16.4 Hz, J_2 = J_3 = 5.4 Hz, 1H), 5.64 (ddd, J = 17.2, 10.3, 8.8 Hz, 1H), 5.24–5.19 (m, 2H), 5.07 (dd, J = 10.3, 1.8 Hz, 1H), 4.87 (d, J = 15.6 Hz, 1H), 4.71 (d, J = 15.6 Hz, 1H), 4.65–4.60 (m, 2H), 4.20 (d, J = 11.9 Hz, 1H), 4.18 (dd, J = 11.2, 4.9 Hz, 1H), 4.11 (sept, J = 6.7 Hz, 1H), 3.76 (dd, J = 11.9, 3.4 Hz, 1H), 3.40 (td, J_1 = J_2 = 12.6 Hz, J_3 = 4.4 Hz, 1H), 2.91–2.80 (m, 3H), 2.77–2.73 (m, 1H), 2.46 (dd, J = 16.2, 2.3 Hz, 1H), 2.33–2.27 (m, 2H), 2.20–2.14 (m, 1H), 2.09–2.02 (m, 2H), 1.88–1.76 (m, 2H), 1.70 (dd, J = 8.2, 5.0 Hz, 1H), 1.55–1.48 (m, 1H), 1.32 (dd, J = 9.2, 5.0 Hz, 1H), 1.17 (d, J = 6.7 Hz, 3H), 1.11 (d, J = 6.7 Hz, 3H), 1.08–0.99 (m, 4H), 0.97 (d, J = 6.8 Hz, 3H), 0.92 (d, J = 6.8 Hz, 3H). LRMS (ESI) m/z calcd for $C_{38}H_{51}N_5O_8S$: 738.3 $[M+1]^+$ found 738.2.
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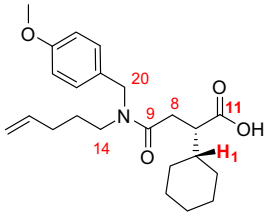
22. Prepared with minor changes and according to the general procedure described in: Dieltiens, N.; Stevens, C. V.; Masschelein, K.; Hennebel, G.; Van der Jeught, S. *Tetrahedron* **2008**, *64*, 4295. ^{1}H NMR (300 MHz, $CDCl_3$, T = 300 K) δ 7.22 (d, J = 8.4 Hz, 2H), 6.85 (d, J = 8.4 Hz, 2H), 5.87–5.73 (m, 1H), 5.03 (br s, 1H), 4.97–4.92 (m, 1H), 3.78 (s, 3H), 3.59 (s, 2H), 2.62 (t, J = 6.9 Hz, 2H), 2.08 (q, J = 7.2 Hz, 2H), 1.65–1.55 (m, 2H).
23. The structure of **22b** (*E*:*Z*-isomers at the amide bond, relative ratio = 1:1.3) was assessed by a ^{1}H–^{13}C HMBC experiment. The two carbonyl carbons of the amide and the acid moieties (detected at 171.1 ppm and 175.3 ppm, respectively) were unambiguously distinguished by the pattern of proton–carbon connectivities observed for the C11–C9 fragment (H8 correlate to both C9 and C11, while H14 and H20 only correlate to C9); the analysis of the proton–carbon connectivities from the c-Hex moiety showed the key correlation H1–C11 (175.3 ppm). The structure of **22a** was assigned in analogous manner.



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26. Compound **2** has a K_i = 0.06 nM and EC_{50} (10% FCS) = 12 nM. The complete characterization of **2** will be the subject of a separate publication by our organization.