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3-Amino-2(5*H*)furanones as inhibitors of subgenomic hepatitis C virus RNA replication

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ABSTRACT

A new class of compounds able to block the replication of subgenomic HCV RNA in liver cells is described. 3-Amino-2(5*H*)furanones **4** may be regarded as diketoacid analogues and were obtained by basic rearrangement of the isoxazolidine nucleus.

1. Introduction

Hepatitis C Virus (HCV) infection constitutes a global health problem, which affects more than 170 million individuals.[1,2] The NS5B RNA-dependent RNA polymerase (NS5B RdRp) has shown to be the catalytic core of the HCV replication machinery.[3,4] This enzyme is not expressed in uninfected cells, and, due to its unique features, represents an attractive target for the development of safe antiviral drugs.[5–7]

The catalytic activity of the enzyme is mediated, in the active site, by two magnesium ions, which serve to activate the 3'-OH of the elongating RNA and to position the incoming nucleotide-triphosphate for the nucleophilic attack.[8–11]

Different classes of NS5B inhibitors have been disclosed and they can be divided by their mechanism of action into three major classes: non-nucleoside inhibitors acting at allosteric binding sites, nucleoside analogues, and pyrophosphate analogues. The allosteric inhibitors include a variety of heterocyclic systems, which have been shown to bind to three distinct sites on the polymerase.[6,7,12]

The others two classes are active-site inhibitors: the first are modified chain-terminating nucleoside (substrate) analogues[13–15] and the second are pyrophosphate (product) analogues,[16,17] namely diketoacids (DKA) **1**. Actually, only three scaffolds have been reported: phenyl-DKA[18] like **1**, meconic acid derivatives **2**[19] and carboxypyrimidines **3**[20] (Fig. 1).

In these last years, we have developed an efficient synthetic procedure towards the construction of 3-amino-2(5*H*)furanones by basic treatment of 3-alkoxycarbonyl substituted isoxazolidines.[21] We have assumed that the introduction of a carbonyl group at the C_4 position of the 3-amino-2(5*H*)furanone skeleton, as in compounds **4**, could produce potential inhibitors of pyrophosphate site. In fact, 3-amino-2(5*H*)furanones **4** may be regarded as DKA cyclic analogues: the 1,3-diketonic functionality is replaced

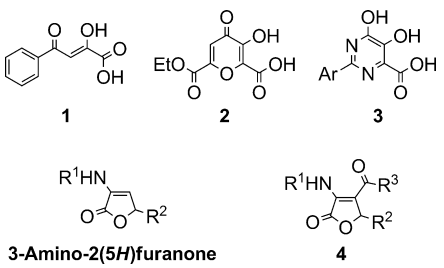

Figure 1. NS5B pyrophosphate analogues inhibitors and new potential ones.

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by the 1-keto-3-imino group, enolized into the corresponding 1-keto-enamino functionality, while the acid moiety is masked in the furanose structure. In this paper we report the synthesis and the anti-HCV activity of 3-amino-2(5*H*)furanones **4**. Some of the newly described compounds have shown a good inhibitory activity in a cell-based subgenomic HCV replication assay.

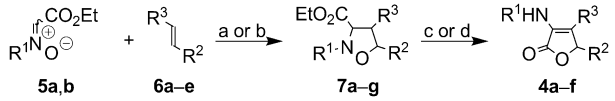
2. Results and discussion

The synthetic scheme toward 3-amino-2(5*H*)furanones **4** relies on the basic treatment of isoxazolidine derivatives **7**, activated at C_3 by the presence of an estereal group, easily accessible by 1,3-dipolar cycloaddition of *C*-alkoxycarbonyl nitrones **5** with suitable alkenes (Scheme 1 and Table 1).

In particular, isoxazolidines **7a–c**,**g**, were prepared by reaction of dipoles **5** with alkenes **6a**–**b**,**e** under microwave irradiation. The cycloaddition reaction proceeded with high yields (85–90%) and the observed regiochemistry and stereochemistry are in agreement with the previously reported results.[22–24] The cycloadducts, obtained as mixture of *cis*/*trans* isomers, were separated by medium pressure liquid chromatography (MPLC).

The classic pericyclic reaction of nitrones with α,β-unsaturated ketones, such as **6c** and **6d**, with or without microwave irradiation affords as major cycloadduct the regioisomer with the ketonic functionality at C_5 position. The regiochemical control towards 4-substituted isoxazolidines **7d** and **7e** was obtained by using the pinhole Lewis acid as catalyst in mild conditions.[25] The reaction of nitrone **5a** with 3-buten-2-one **6c** shows a dramatic change of regioselectivity between the catalyzed and uncatalyzed reactions. In fact, while, without catalyst the only C_5 substituted compound was obtained with a 60% yield, in the presence of aluminum tris(2,6-diphenylphenoxide) (ATPH) catalyst the crude reaction mixture showed the only desired C_4 substituted compound with a 85% yield. Moreover, isoxazolidine **7d** was obtained as single stereoisomer. ATPH is known to give stable complexes with carbonyl compounds such as alkenes **6c** and **6d**, so inducing on the dipolarophile an electron withdrawing effect at the double bound and a steric bulk at the carbonyl functionality.[25] As a consequence of these effects, the expected major cycloadduct arises from a reversed regiochemistry with the presence of a carbonyl group at C_4 position of the isoxazolidine nucleus. The reaction of nitrone **5a** with 3-penten-2-one **6d**, in the presence of ATPH, shows a minor control of regioselectivity where the ratio **7e/7f** is 4:1 (80% yield, see Section 4). The obtained four cycloadducts were separated by MPLC and the relative stereochemical assignment was performed by NOE measurements.

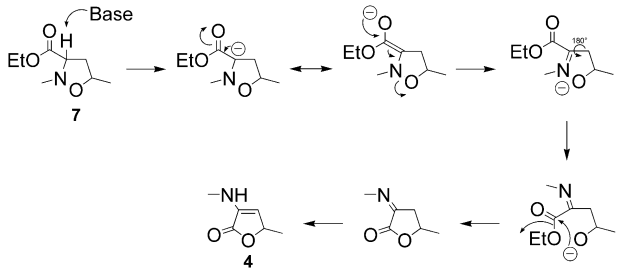
The chemical conversion of 3-alkoxycarbonyl isoxazolidines **7**, as mixture of *cis* and *trans* isomers, into 3-methylamino-2-(5*H*)furanones **4** have been performed using a mild base, such as the tetrabutyl ammonium fluoride (TBAF) (75–85% yield). For C_4 carbonyl substituted isoxazolidines the use of NaH, as previously reported,[21] promote a competitive side reaction leading to degradation products not easily characterizables. The driving force for this rearrangement is represented by the low critical energy required to induce an ionic centre at C_3 position of the isoxazolidine nucleus which promotes the ring opening of the heterocyclic system and the subsequent intramolecular lactonization (Scheme 2).



Scheme 1. Reagents and conditions: (a) Microwaves, toluene, 100 W, 80 °C, 30 min; (b) DCM dry, ATPH 10 mol%, 0 °C, 12 h; (c) THF dry, TBAF, reflux 6 h; (d) THF dry, NaH, rt, 4 h.

Table 1
Substituents on compounds **4–7**

Compound	R^1	R^2	R^3
5a	Me	—	—
5b	Bn	—	—
6a	—	CO_2Me	Me
6b	—	CO_2Et	CO_2Et
6c	—	H	C(O)Me
6d	—	Me	C(O)Me
6e	—	Ph	Ph
7a	Me	Me	CO_2Me
7b	Bn	Me	CO_2Me
7c	Me	CO2Et	CO_2Et
7d	Me	H	C(O)Me
7e	Me	Me	C(O)Me
7f	Me	C(O)Me	Me
7g	Me	Ph	Ph
4a	Me	Me	CO_2Me
4b	Bn	Me	CO_2Me
4c	Me	CO_2Et	CO_2Et
4d	Me	H	C(O)Me
4e	Me	Me	C(O)Me
4f	Me	Ph	Ph

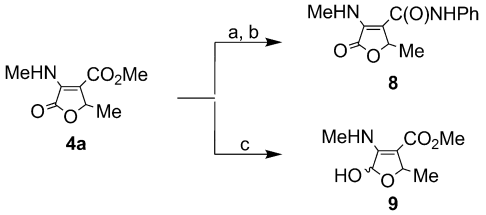


Scheme 2. Chemical conversion of the isoxazolidine nucleus to 3-amino-2(5*H*)furanone.

Compounds **8** and **9**, useful for our biological studies were obtained starting from compound **4a** (Scheme 3). Thus, the *N*-phenyl carboxamide **8** was obtained by basic treatment of **4a** with potassium carbonate followed by reaction with aniline in the presence of *N*,*N*-diisopropilcarbodiimide, while the desired lactol **9** was obtained by DIBAL-H reduction of **4a**, in quantitative yield.

The anti-HCV activity of all the synthesized compounds has been tested directly in a cell-based subgenomic HCV replicon system. Thus, the inhibition of replication of HCV RNA was measured in Huh-7-derived HBI10A cells, harboring a subgenomic HCV replicon using a cell-based assay, as previously described.[26,27] The screening of all the compounds was performed up to the fixed concentration of 10^3 μM. The inhibitory activity of compounds **4a–f**, **8** and **9**, expressed as EC_{50}, and the relative toxicity, expressed as CC_{50}, are reported in Table 2 besides the respective Pearson's *r* values.

The comparison of EC_{50} of **4a** (EC_{50} = 525 μM), **4e** (EC_{50} = 132 μM), and **8** (EC_{50} = 19 μM) shows that by replacing the ke-



Scheme 3. Reagents and conditions: (a) MeOH, K_2CO_3; (b) DMF dry, aniline, *N*,*N*-diisopropilcarbodiimide, DIEA, rt 5 h; (c) Et_2O dry, DIBAL-H, −78 °C, 5 h.

Table 2
Inhibitory activity of compounds **4a–f**, **8** and **9**, expressed in EC_{50}, relative toxicity, expressed in CC_{50}, and the respective Pearson's *r* values

Compound	EC_{50} (μM)	Pearson's *r*	CC_{50} (μM)	Pearson's *r*
4a	525	0.92	>1000	—
4b	>1000	—	>1000	—
4c	>1000	—	>1000	—
4d	521	0.81	>1000	—
4e	132	0.82	819	0.89
4f	17	0.89	28	0.88
8	19	0.95	>1000	—
9	66	0.93	>1000	—

tone functionality with an estereal or an amide ones, the biological activity changes in the order C(O)NHR > C(O)R > C(O)OR.

From these data the amide **8** emerges as the more active compound and its EC_{50} is comparable with that of 5,6-dihydroxy-2-(2-thienyl)pyrimidine-4-carboxylic acid (EC_{50} = 9.3 μM; IC_{50} = 0.15 μM), which at the best of our knowledge is the more active pyrophosphate inhibitor reported in literature.[20] Moreover, a CC_{50} value of >1000 μM ensures to this compound a very high selectivity index. The results in Table 2 suggest that R^2 = methyl is an important requisite for the biological activity, since its substitution with a hydrogen atom such as in compound **4d** (EC_{50} = 521 μM) or ethoxycarbonyl as in **4c** (no active) induces a significative loss of activity.

When phenyl groups are present at C_5 and C_4 of furanone nucleus, **4f** (EC_{50} = 17 μM; CC_{50} = 28 μM), the toxicity of this compound prevents the evaluation of the effect of the aryl substituents on the activity. The substitution of the methyl group at the nitrogen atom (R^1) with a benzyl group leads to a complete lack of activity (see Table 2).

We have also investigated the effect of modifications on the 2(5*H*)furanone skeleton. Thus, lactol **9**, chosen as model compound, shows an EC_{50} of 66 μM. This modification has produced a 8-fold improvement in potency with respect to **4a**.

The biological activity of DKA as inhibitors of NS5B polymerase is related to their ability to chelate Mg^{2+} and Mn^{2+} ions; however, the recent characterization of the affinity of the enzyme for metal ions suggests that magnesium is the cation that is used in vivo during polymerization.[28] On the basis of the considerations that our 3-amino-2(5*H*)furanones can be regarded as DKA mimetics, we have investigated their ability to complex Mg^{2+} ions by semiempirical calculations. Although there are two magnesium ions in the active site, a catalytic mechanism has been proposed for polymerases in which one metal ions is involved in both positioning the substrate and in the activation of an incoming nucleophile.[29] Nucleophilic attack would then generate a trigonal bipyramidal transition state that would be stabilized by both metal ions. The second metal ion also stabilizes the negative charge that appears on the leaving 3'-oxygen, thus facilitating its departure from the phosphate. On these bases we have conducted an in silico study upon the formation and the stabilities of the 1:1 complexes generated by compounds **4a–e**, **8** and **9** with Mg^{2+} ion. These complexes can be produced in two possible modes, complex-1 (C1) and complex-2 (C2), corresponding to the two different sites of complexation (Fig. 2).

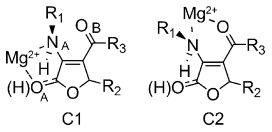


Figure 2. The two possible 1:1 Mg^{2+} complexes for compounds **4a–e**, **8** and **9**.

To obtain the relative stability for each type of complex we have calculated the enthalpies for the complexation reaction, as indicated in Eq. (1).

$$\Delta H_{(\text{react.})} = H_{f(\text{complex})} - [H_{f(\text{isolated form of the ligand})} + H_{f(Mg^{2+})}] \quad (1)$$

All the calculations were performed utilizing the new PM6 semiempirical hamiltonian[30] as implemented in MOPAC 2007 package[31] using Winmostar as GUI interface.[32] In all the cases, full geometry optimization was carried out without any symmetry constraints.

The obtained results, reported in Table 3, show that in all the cases the more stable complex, within each compound, arises from the interaction of Mg^{2+} with the lone pairs of the enamine nitrogen and of the oxygen of the carbonyl group at C_4, corresponding to the complex **2** site. The formation of a cyclic six member complex is always favoured upon the five membered one. Moreover, for compounds **4a**, **4e** and **8**, which differ for the R_3 substituent, the stability of Mg^{2+} complex (C2) follows the trend **8** > **4e** > **4a**, in complete agreement with the biological results, i.e., the activity of compounds is directly proportional to the ability of magnesium complexation.

This trend is also supported by a major electron availability on O_B (see Fig. 2 and Table 3) ongoing from ester to amide functionalities. The better value of the complexation energy showed by compound **4b**, which does not exhibit any biological activity can be ascribed to the net π-Mg^{2+} stabilizing interaction due to the phenyl present into the *N*-benzyl substituent; however, it is likely to consider that this conformation is precluded in the enzymatic site for steric hindrance.

According to the examination of complexation Δ*H* values, compound **4c** should show a biological activity similar to that of **4a** and **4d**. However the observed lack of antiviral activity of **4c** ($R^1 = R^2 = CO_2Et$) suggested that, besides the complexation energy, hydrophobic effect plays an important role with the preference of a hydrophobic substituent at C_5 with respect to a polar one.

Finally, the gain in stability for the **9**-C2, if compared to **4a**-C2, arises from the increasing of negative charges on both N_A and O_B atoms due to the loss of the attractive conjugation with the lactone oxygen.

Table 3
Enthalpies of formation (kcal/mol) for C1 and C2 Mg^{2+} complexes of compounds **4a–e**, **8** and **9**, and O_A, O_B and N_A atom charges in not complexed compounds

Compound[a]	H_f	$H_{f(C1)}$	$H_{f(C2)}$	$\Delta H_{(\text{react.})}$ for C1	$\Delta H_{(\text{react.})}$ for C2	O_A q(e)	O_B q(e)	N_A q(e)
4a	−161.58	240.38	236.27	−141.13	−145.24	−0.45	−0.59	−0.25
4b	−136.55	224.31	220.60	−182.23	−185.94	−0.45	−0.59	−0.28
4c	−229.92	177.16	169.77	−136.01	−143.40	−0.41	−0.59	−0.23
4d	−110.97	292.56	289.61	−139.56	−142.51	−0.44	−0.59	−0.25
4e	−119.95	280.19	277.86	−142.95	−145.28	−0.45	−0.60	−0.25
8	−92.12	303.81	289.78	−147.16	−161.19	−0.44	−0.61	−0.25
9	−176.86	218.08	212.35	−148.15	−153.88	−0.57	−0.61	−0.28

[a] $H_{f(Mg2+)}$ = 543.09 kcal/mol.

3. Conclusions

The synthesis and the biological activity of a new class of compounds, inhibitors of subgenomic HCV RNA in the replicon assay, have been reported. From this study, 2,5-dihydro-2-methyl-4-(methylamino)-5-oxo-*N*-phenylfuran-3-carboxamide **8** emerges as the most active compound and its EC_{50} is comparable with that of 5,6-dihydroxy-2-(2-thienyl)pyrimidine-4-carboxylic acid (EC_{50} = 9.3 μM)[20] which, at the best of our knowledge, is the more active pyrophosphate inhibitor in the replicon assay.

4. Experimental

4.1. General

Solvents and reagents were used as received from commercial sources. Melting points were determined with a Kofler apparatus and are reported uncorrected. Elemental analysis was performed with a Perkin-Elmer elemental analyzer. Nuclear magnetic resonance spectra (^{1}H NMR recorded at 300 or 500 MHz, ^{13}C NMR recorded at 75 or 125 MHz) were obtained on Varian Instruments and are referenced in ppm relative to TMS or the solvent signal. Thin-layer chromatographic separations were performed on Merck silica gel 60-F_{254} precoated aluminum plates. Flash chromatography was accomplished on Merck silica gel (200–400 mesh). Preparative separations were carried out by MPLC Büchi C-601 using Merck silica gel 0.040–0.063 mm and the eluting solvents were delivered by a pump at the flow-rate of 3.5–7.0 mL min^{-1}. The reaction under microwave irradiation was carried out using a CEM Corp. Focused Microwave System, Model Discover. The identification of samples from different experiments was secured by mixed melting points and superimposable NMR spectra.

The following compounds were prepared according to described procedures: (*E*)- and (*Z*)-*C*-ethoxycarbonyl-*N*-methyl nitrone **5a** and (*E*)- and (*Z*)-*C*-ethoxycarbonyl-*N*-benzyl nitrone **5b**.[27]

4.2. General procedure for the synthesis of isoxazolidines 7a–c, 7g

A solution of nitrone **5** (0.5 mmol) and alkene **6** (1.5 mmol) in dry toluene (5 mL) in a pressure tube equipped with a stir bar was inserted into the cavity of a discover Microwave System apparatus and heated at 90 W, 80 °C, for 20–30 min. The mixture was evaporated and the resulting solid was purified by MPLC on a silica gel with cyclohexane/ethyl acetate (80:20).

4.2.1. 3-Ethoxycarbonyl-4-methoxycarbonyl-2,5-dimethyl isoxazolidine (7a)

The synthesis of compounds **7a** as *cis*/*trans* mixture, started from *N*-methyl-*C*-ethoxycarbonyl nitrone **5a** and methyl crotonate **6a**. The analytical and spectroscopical data were previously reported.[22]

4.2.2. 3-Ethoxycarbonyl-4-methoxycarbonyl-2-benzyl-5-methyl isoxazolidine (7b)

The synthesis of compounds **7b** as *cis*/*trans* mixture, started from *N*-benzyl-*C*-ethoxycarbonyl nitrone **5b** and methyl crotonate **6a**. The analytical and spectroscopical data were previously reported.[23]

4.2.3. 3-Ethoxycarbonyl-4,5-dimethoxycarbonyl 2-methyl isoxazolidine (7c)

The synthesis of compounds **7a** as *cis*/*trans* mixture, started from *N*-methyl-*C*-ethoxycarbonyl nitrone **5a** and dimethyl fumarate **6b**. The analytical and spectroscopical data were previously reported.[23]

4.2.4. 3-Ethoxycarbonyl-4,5-diphenyl-2-methyl isoxazolidine (7g)

The synthesis of compounds **7a** as *cis*/*trans* mixture, started from *N*-methyl-*C*-ethoxycarbonyl nitrone **5a** and *trans* stilbene **6e**. The analytical and spectroscopic data were previously reported.[23]

4.3. General procedure for the synthesis of isoxazolidines 7d–f

To a solution of 2,6-diphenylphenol (290 mg, 0.38 mmol) in dry dichloromethane (20 mL) at 0 °C was added under N_2 atmosphere trimethylaluminum (0.2 mL, 2M solution in toluene) and the solution was left stirring for 30 min at this temperature. Then, 3.8 mmol of alkene **6c** or **6d** was added at 0 °C and, after 30 min, a solution of nitrone **6a** (3.8 mmol in 10 mL of CH_2Cl_2) was added dropwise during 20 min. The reaction mixture was stirred for 12 h at room temperature. Then, the mixture was filtered on a Celite pad, the filtrate was evaporated in vacuo and the residue subjected to MPLC chromatography.

The reaction of *N*-methyl-*C*-ethoxycarbonyl nitrone **5a** and but-3-en-2-one **6c** affords **7d** as single stereoisomer (yield 85%).

4.3.1. (3*RS*,4*SR*)-3-Ethoxycarbonyl-4-acetyl-2-methyl isoxazolidine (7d)

Light yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.27 (t, 3H, J = 7.1 Hz), 2.25 (s, 3H), 2.78 (s, 3H), 3.74 (d, 1H, J = 5.4), 3.87 (ddd, 1H, J = 5.0, 5.4 and 8.7 Hz), 4.13 (dd, 1H, J = 5.0 and 8.7 Hz), 4.17 (dd, 1H, J = 8.7 and 8.5 Hz), 4,22 (q, 2H, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.0, 28.4, 42.9, 59.4, 61.7, 67.4, 70.1, 171.5, 194.0. HRMS Calcd for (M^+) $C_9H_{15}NO_4$: 201.1001. Found: 201.1005.

The reaction of *N*-methyl-*C*-ethoxycarbonyl nitrone **5a** and pent-3-en-2-one **6d** affords the regioisomers **7e** and **7f** as a mixture of two *cis*/*trans* isomers (global yield 80%).

4.3.2. 3-Ethoxycarbonyl-4-acetyl-2,5-dimethyl isoxazolidine (7e)

First eluted compound; yield 43%, light yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1,29 (t, 3H, J = 7.1 Hz), 1.43 (d, 3H, J = 6.3 Hz), 2.20 (s, 3H), 2.80 (s, 3H), 3.45 (dd, 1H, J = 5.0 and 7.5 Hz), 3.49 (d, 1H, J = 5.0 Hz), 4.20 (q, 2H, J = 7.1 Hz), 4.21 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.5, 17.2, 28.9, 43.3, 61.5, 62.0, 65.6, 71.2, 170.0, 210.0. HRMS Calcd for (M+) $C_{10}H_{17}NO_4$: 215.1158. Found: 215.1155.

Second eluted compound; yield 21%, light yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1,32 (t, 3H, J = 7.2 Hz), 1.40 (d, 3H, J = 6.1 Hz), 2.20 (s, 3H), 2.81 (s, 3H), 3.45 (d, 1H, J = 5.0 Hz), 3.55 (dd, 1H, J = 5.0 and 6.1 Hz), 4.25 (q, 2H, J = 7.2 Hz), 4.40 (quintet, 1H, J = 6.1 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.7, 17.5, 30.0, 43.5, 61.6, 62.0, 65.6, 70.5, 169.5, 210.0. HRMS Calcd for (M+) $C_{10}H_{17}NO_4$: 215.1158. Found: 215.1156.

4.3.3. 3-Ethoxycarbonyl-5-acetyl-2,4-dimethyl isoxazolidine (7f)

First eluted compound: yield 8%; yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.25 (d, 3H, J = 6.9 Hz), 1.31 (d, 3H, J = 7.1 Hz), 2,30 (s, 3H), 2.85 (s, 3 H), 2.86 (d, 1H, J = 6.1 Hz), 2.90 (m, 1H), 4.12 (d, 1H, J = 6.9 Hz), 4.30 (q, 2H, J = 7.1 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.9, 15.2, 25.5, 34.2, 43.5, 61.5, 65.4, 98.2, 171.0, 208.5. HRMS Calcd for (M+) $C_{10}H_{17}NO_4$: 215.1158. Found: 215.1160.

Second eluted compound: yield 8%; yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1,30 (d, 3H, J = 7.1 Hz), 1.32 (d, 3H, J = 8.7 Hz), 2.30 (s, 3H), 2.82 (s, 3H), 2.85 (d, 1H, J = 6.7 Hz), 2.95 (m, 1H), 3.85 (d, 1H, J = 8.7 Hz), 4,22 (q, 2H, J = 7.1 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.5, 15.2, 25.3, 33.7, 43.2, 61.7, 65.3, 99.0, 171.1, 208.1. HRMS Calcd for (M+) $C_{10}H_{17}NO_4$: 215.1158. Found: 215.1155.

4.4. General procedure for the synthesis of 3-amino-2(5*H*)furanones 4a–e

Method A. To a solution of isoxazolidine **7a**–**e** (1 mmol) in dry THF (10 mL) was added TBAF (1.1 mL, 1.1 mmol, 1M in THF) and the mixture was stirred at 50 °C for 6 h. At the end of this time, the solvent was removed and the residue was purified by MPLC using $CHCl_3$/MeOH (99:1) as eluent.

Method B. To a solution of isoxazolidine **7f** (1 mmol) in dry THF (10 mL) was added NaH (24 mg, 1 mmol) and the mixture was stirred for 4 h at room temperature. The reaction mixture was then quenched with water (0.5 mL) and evaporated under reduced pressure. The residue was purified by MPLC using $CHCl_3$/MeOH (99:1) as eluent.

4.4.1. 5-Methyl-4-methoxycarbonyl-3-methylamino-2(5*H*)furanone (4a)

Yield 85%; white solid, mp 80–82 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.35 (d, 3H, J = 7.1 Hz), 3.25 (d, 3H, J = 6.2 Hz), 3.77 (s, 3H), 5.10 (q, 1H, J = 6.1 Hz), 6.20 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.1, 29.8, 51.0, 75.1, 109.4, 142.1, 165.0, 167.5. HRMS Calcd for (M^+) $C_8H_{11}NO_4$:185.0688. Found: 185.0691.

4.4.2. 5-Methyl-4-methoxycarbonyl-3-benzylamino-2(5*H*)furanone (4b)

Yield 80%; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1. 45 (d, 3H, J = 6.2 Hz), 3.80 (s, 3H), 4.95 (dd, 1H, J = 6.1 and 12.5 Hz), 5.05 (dd, 1H, J = 6.1 and 12.5 Hz), 5.20 (q, 1H, J = 6.2 Hz), 6.95 (br s, 1H, NH) 7.30 (m, 5H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.2, 46.3, 51.3, 61.2, 87.3, 112.1, 126.9, 128.1, 129.2, 138.7, 165.0, 167.4. HRMS Calcd for (M^+) $C_{14}H_{15}NO_4$: 261.1001. Found: 261.1006.

4.4.3. 4,5-Diethoxycarbonyl-3-methylamino-2(5*H*)furanone (4c)

Yield 79%; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.30 (t, 3H, J = 7.1 Hz), 1.38 (t, 3H, J = 7.2 Hz), 3.05 (d, 3H, J = 5.2 Hz), 4.14 (q, 2H, J = 7.1 Hz), 4.25 (q, 2H, J = 7.2 Hz), 5.10 (s, 1H), 8.02 (br s, NH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 13.7, 13.8, 29.7, 57.4, 61.8, 86.7, 124.9, 129.1, 168.0, 70.2, 171.5. HRMS Calcd for (M^+) $C_{11}H_{15}NO_6$: 257.0899. Found: 257.0894.

4.4.4. 4-Acetyl-3-methylamino-2(5*H*)furanone (4d)

Yield 82%; light yellow solid; mp 120–124 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.13 (s, 3H), 3.30 (d, 3H, J = 5.7 Hz), 4.96 (s, 2H), 7.94 (br s, NH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 26.7, 27.3, 67.7, 121.0, 158.7, 175.3, 202.1, HRMS Calcd for (M^+) $C_7H_9NO_3$: 155.0582. Found: 155.0579.

4.4.5. 5-Methyl-4-acetyl-3-methylamino-2(5*H*)furanone (4e)

Yield 75%; light yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.57 (d, 3H, J = 6.3 Hz), 2.20 (s, 3H), 3.35 (d, 3H, J = 5.5 Hz), 5.3 (q, 1H, J = 6.3 Hz), 8.3 (br s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.4, 26.8, 45.2, 75.9, 117.9, 142.5, 167.0, 193.1. HRMS Calcd for (M^+) $C_8H_{11}NO_3$: 169.0739. Found: 169.0735.

4.4.6. 4,5-Diphenyl-3-methylamino-2(5*H*)furanone (4f)

Yield 78%; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.61 (s, 3H), 4.12 (br s, 1H), 5.95 (s, 1H), 7.10–7.30 (m, 10H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 32.0, 83.7, 94.5, 124.6, 127.6, 127.7, 128.0, 128.6, 128.8, 129.0, 131.1, 132.2, 136.1, 171.0. HRMS Calcd for (M^+) $C_{17}H_{15}NO_2$: 265.1103. Found: 265.1105.

4.5. Synthesis of 2,5-dihydro-2-methyl-4-(methylamino)-5-oxo-*N*-phenylfuran-3-carboxamide (8)

Compound **4a** (205 mg, 1.1 mmol) was dissolved in methanol (3 mL) and treated with a 10% aqueous solution of potassium carbonate (3 mL). The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH 7:3). The mixture was neutralized with 2 N HCl and then evaporated in vacuo. The crude was treated with chloroform and the organic extracts were concentrated under reduced pressure.

To a solution of the crude material in dry DMF (3 mL) and aniline (103 mg, 1 mmol) were added *N*,*N*-diisopropilcarbodiimide (500 mg, 13 mmol) and DIEA (500 μL, 3 mmol) at room temperature. The reaction mixture was stirred for 5 h and then was quenched by the addition of brine and extracted three times with EtOAc. The organic layers were dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by MPLC using ethyl acetate/cyclohexane 4:6 as eluent to give **8** as yellow oil (yield 85%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.45 (s, 3H), 1.62 (d, 3H, J = 8.5 Hz), 3.21 (d, 3H, J = 7.4 Hz), 5.30 (q, 1H, J = 8.5 Hz), 6.80 (br s, 1H), 6.95 (br s, 1H), 7.20–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.3, 29.9, 74.5, 106.4, 120.4, 124.8, 126.6, 129.1, 154.3, 162.8, 168.1. HRMS Calcd for (M^+) $C_{13}H_{14}N_2O_3$: 246.1004. Found: 246.1008.

4.6. Synthesis of Methyl 2,5-dihydro-5-hydroxy-2-methyl-4-(methylamino)furan-3-carboxylate (9)

To a solution of **4a** (185 mg, 1 mmol) in anhydrous ether (30 mL) at −78 °C, under nitrogen atmosphere a solution of DIBAL-H (2 mL, 2 mmol, 1M) was added and the mixture was stirred at the same temperature for 5 h. The solution was then quenched with MeOH (1 mL) and water (1 mL) and the resulting precipitate was filtered under reduced pressure. The filtrate was evaporated in vacuo to obtain an inseparable mixture of α- and β-anomers of lactol **9** in 1:1.2 ratio and in a quantitative yield as yellow oil. Major anomer: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.22 (d, 3H, J = 7.2 Hz), 3.10 (d, 3H, J =4.9 Hz), 3.79 (s, 3H), 5.20 (dq, 1H, J = 0.5 and 7.2 Hz), 5.95 (d, 1H, J = 0.5 Hz), 6.70 (br s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 22.2, 30.5, 50.5, 79.2, 97.5, 109.5, 158.6, 173.0. Minor anomer: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.35 (d, 3H, J = 7.1 Hz), 3.10 (d, 3H, J = 4.9 Hz), 3.79 (s, 3H), 4.97 (q, 1H, J = 7.1 Hz), 6.05 (br s, 1H), 6.70 (br s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 24.4, 29.7, 50.1, 79.9, 98.0, 109.5, 158.6, 166.3.

4.7. Biological assays

4.7.1. Cell culture

Huh-7 cells, originally obtained from Ralf Bartenschlager (University of Mainz, Mainz, Germany) were grown in Dulbecco's modified minimal essential medium (D-MEM, EuroClone, Pero, Italy), supplemented with 10% fetal bovine serum (FBS, Life Technologies, Paisley, Scotland, UK). Huh-7-derived HBI10A cells expressing an HCV subgenomic replicon have been previously described.[26] They were grown as described for Huh-7 cells, but the medium was supplemented with the addition of 0.8 mg of neomycin sulfate (G418, Life Technologies). Cells were passaged 1:5 twice a week using 1× trypsin–EDTA.

4.7.2. Anti-hepatitis C virus assay

The effect of compounds on HCV viral replication was monitored in HBI10A cells by a cell-enzyme-linked immunosorbent assay, as previously described.[27] Briefly, HBI10A cells, either treated with different concentrations of the compounds or control diluent, were assayed for NS3 protein expression with the anti-NS3 10E5/24 MAb. Compounds were dissolved in DMSO (Sigma Chemicals CO., St. Louis, MO) and serially diluted in D-MEM in a way that DMSO concentration was never higher than 1%. Final concentrations of the compounds were 10^3, 10^2, 10, and 1 μM. The assay was performed in triplicate. As a positive control, IFN-α at concentrations ranging from 10^2 to 1 U/mL, was utilised. The inhibitor

concentration that reduced by 50% the expression of NS3 (EC_{50}) was calculated by fitting the data to the Hill equation: fraction inhibition = $1 - (A_i - b)/(A_0 - b) = [I]^n/([I]^n + EC_{50})$, where A_i is the absorbance value of HBI10A cells supplemented with the appropriate compound [I] concentration, A_0 is the absorbance value of HBI10A cells incubated with control diluent, b is the absorbance value of Huh-7 cells plated at the same density in the same microtiter plates and incubated with control diluent, and n is the Hill coefficient. The EC_{50} values were calculated according to the best-fit curve, y value versus $\log x$, where y is the value of the examined function and x is the drug concentration. The Pearson product–moment correlation coefficient (Pearson's r), that reflects the degree and direction of linear relationship between two variables, was also calculated for each significant value of EC_{50}.

4.7.3. Cytotoxicity assay

Cytotoxicity of the compounds on HBI10A cells was detected by a MTS assay. HBI10A cells were seeded at 1×10^4 cell/100 μL of D-MEM + 5% FBS in a 96 well plate and after 4 h incubation at 37 °C cells the compounds were added at the final concentration of 10^3,10^2, 10 and 1 μM. After 20 h incubation at 37 °C, 20 μL of MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] were added to each well. Samples were then incubated for a further 4 h at 37 °C before the reaction was stopped through the addition of 20 μL SDS, 10% SDS and the absorbance was measured at 492 nm. Each condition was analysed in triplicate.

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