Novel Peptide Mimetic Inhibitors of Hepatitis C Serine Protease Derived from Isomannide

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Abstract: Hepatitis C (HCV) infection is a cause of chronic liver disease such as cirrhosis, carcinoma, or liver failure, and the current therapy is effective in only 50% of patients. Serine proteases, which are present in HCV, are the most studied class of proteolytic enzymes, and are a primary target in the drug development field. In this paper, we describe the synthesis and biological studies of a novel class of peptide mimetic compounds as potential HCV serine protease inhibitors.

Key words: peptides, antiviral agent, fused-ring systems, isomannide, hepatitis C

More than 170 million people worldwide are affected by the hepatitis C virus (HCV). HCV infection is a leading cause of chronic liver disease such as cirrhosis, carcinoma, or liver failure.1 The current pegylated interferon and ribavirin combination therapy is effective in only 50% of patients. Its moderate efficacy and apparent side effects underscore the need for safer and more effective treatments.2 HCV NS3 protease is a serine protease presenting a characteristic catalytic triad (His51, Asp75, and Ser135) conserved in all flaviviruses.3,4 The NS3pro activity is essential for viral replication, representing a suitable target for the development of new medicines in the treatment of hepatitis C. However, the lack of a robust in vitro cell culture system and the absence of a convenient small animal model have hampered the assessment of both in vitro and in vivo efficacy of any antiviral compounds.

As part of our antiviral program for flaviviruses, we describe here the synthesis and biological studies of a series of peptide mimetic compounds designed as potential inhibitors of HCV serine protease.

Studies on the development of peptide mimetic inhibitors have demonstrated the importance of the peptide approach in drug research. Compounds presenting a fused bicyclic structure, such as Danuravir (TMC-114)5 and VX-950,6 represent an effort toward the design and development of new drugs.

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We envisaged the use of the isomannide rigid scaffold7 by its structural analogy with cyclic rigid dipeptides.8 So the rigidity of this scaffold would fix a conformation of our peptide mimetic compound as demonstrated previously for our esters and amides possessing C3 symmetry.

The first step of our synthesis consisted in the transformation of isomannide (1) to its monotosyl derivative 2 using tosyl chloride in pyridine. The crude product, which consisted mainly of the monotosylate 2, was purified by chromatography on silica gel (hexane–EtOAc). Isomannide monotosylate 2 was then O-alkylated under phase-transfer conditions with benzyl chloride in 50% aqueous sodium hydroxide affording the product, O-benzylated 3.9,10 Several solvents can be used in the nucleophilic substitution step, such as N,N-dimethylformamide and dimethyl sulfoxide. However, the ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate {[bmim]BF4}−, shows different advantages over the organic solvents, as well as having improved the reaction yield. The ionic liquid {[bmim]BF4}− was synthesized following the methodology described in literature.11,12 Ionic solvents possess several properties, such as low melting point, negligible vapor pressure, low coordinating ability, and excellent thermal and chemical stability, which make them attractive alternatives to traditional solvents; for these reasons they are called ‘green’ solvents.13 The S2,2-type reaction of 3 with sodium azide was performed in ionic liquid, {[bmim]BF4}−, yielding the azido derivative 4 with inversion of configuration. The azido reduction of 4 with hydrogen over palladium-on-carbon gave the amino derivative 5 in quantitative yield (Scheme 1).14–16

Initially, the oxazolones (so-called azalactones) 8a–o were synthesized from commercially available glycine (6) and benzoyl chloride or acetic anhydride obtaining the N-benzyloxyglycine (7a) and N-acetylglucine (7b), which reacted with different aldehydes in presence of anhydrous sodium acetate/acetic anhydride by Erlenmeyer conditions;17,18 this methodology gives only the thermodynamically stable Z-isomer (Scheme 2).19,20 The last step consisted of the ring opening of the oxazolones 8a–o, obtained via Erlenmeyer synthesis, by the amine 5 yielding the final peptide mimetic products 9a–o (Scheme 3).21–23
It is worth pointing out that although most of the oxazolones 8 used to produce our target compounds 9 have already been reported, compounds 9a–o described in Table 1 have not been previously reported in the literature, which, of course, emphasizes the novelty of the results described herein.

Subgenomic HCV replicon cell culture systems have significantly impacted the field of HCV research and anti-HCV drug discovery.\textsuperscript{32,33} HCV replicon-based assay has been previously reported as sensitive and specific for anti-

Scheme 1 Synthesis of O-benzylated amino derivative 5

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{COOH} \\
& \quad \text{BzCl, 10% NaOH} \\
& \quad \text{HCl or Ac}_2\text{O, H}_2\text{O} \\
& \quad 95\% \text{ or } 77\% \\
\end{align*}
\]

Scheme 2 Synthesis of oxazolone derivatives 8a–o using Erlenmeyer conditions

It is worth pointing out that although most of the oxazolones 8 used to produce our target compounds 9 have already been reported, compounds 9a–o described in Table 1 have not been previously reported in the literature, which, of course, emphasizes the novelty of the results described herein.

Subgenomic HCV replicon cell culture systems have significantly impacted the field of HCV research and anti-HCV drug discovery.\textsuperscript{32,33} HCV replicon-based assay has been previously reported as sensitive and specific for anti-

Table 1 Oxazolones 8 and Peptidemimetic Compounds 9

<table>
<thead>
<tr>
<th>Oxazolone</th>
<th>R\textsuperscript{1}</th>
<th>R\textsuperscript{2}</th>
<th>Mp (°C)</th>
<th>Yield (%) of 8</th>
<th>Peptide mimetic</th>
<th>Yield (%) of 9</th>
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</thead>
<tbody>
<tr>
<td>8a</td>
<td>Ph</td>
<td>Ph</td>
<td>169–170</td>
<td>61</td>
<td>9a</td>
<td>50</td>
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<tr>
<td>8b</td>
<td>Ph</td>
<td>4-FC\textsubscript{6}H\textsubscript{4}</td>
<td>186</td>
<td>71</td>
<td>9b</td>
<td>55</td>
</tr>
<tr>
<td>8c</td>
<td>Ph</td>
<td>4-CIC\textsubscript{6}H\textsubscript{4}</td>
<td>199–200</td>
<td>60</td>
<td>9c</td>
<td>60</td>
</tr>
<tr>
<td>8d</td>
<td>Ph</td>
<td>4-BrC\textsubscript{6}H\textsubscript{4}</td>
<td>207–208</td>
<td>66</td>
<td>9d</td>
<td>55</td>
</tr>
<tr>
<td>8e</td>
<td>Ph</td>
<td>4-F\textsubscript{6}CC\textsubscript{6}H\textsubscript{4}</td>
<td>174–175</td>
<td>78</td>
<td>9e</td>
<td>50</td>
</tr>
<tr>
<td>8f</td>
<td>Ph</td>
<td>4-MeOC\textsubscript{6}H\textsubscript{4}</td>
<td>162–162</td>
<td>55</td>
<td>9f</td>
<td>57</td>
</tr>
<tr>
<td>8g</td>
<td>Ph</td>
<td>2-thienyl</td>
<td>176–178</td>
<td>84</td>
<td>9g</td>
<td>60</td>
</tr>
<tr>
<td>8h</td>
<td>Ph</td>
<td>3,4-(OCH\textsubscript{2}O)C\textsubscript{6}H\textsubscript{3}</td>
<td>200</td>
<td>50</td>
<td>9h</td>
<td>60</td>
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<tr>
<td>8i</td>
<td>Ph</td>
<td>3-pyridyl</td>
<td>154</td>
<td>40</td>
<td>9i</td>
<td>62</td>
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<tr>
<td>8j</td>
<td>Ph</td>
<td>2-furyl</td>
<td>169–171</td>
<td>55</td>
<td>9j</td>
<td>50</td>
</tr>
<tr>
<td>8k</td>
<td>Me</td>
<td>2-thienyl</td>
<td>120</td>
<td>55</td>
<td>9k</td>
<td>50</td>
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<tr>
<td>8l</td>
<td>Ph</td>
<td>1-benzothiophen-2-yl</td>
<td>200</td>
<td>64</td>
<td>9l</td>
<td>40</td>
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<tr>
<td>8m</td>
<td>Ph</td>
<td>2-naphthyl</td>
<td>150–152</td>
<td>40</td>
<td>9m</td>
<td>67</td>
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<tr>
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<td>Ph</td>
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<td>205–207</td>
<td>40</td>
<td>9n</td>
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<td>8o</td>
<td>Ph</td>
<td>benzofuran-2-yl</td>
<td>172–173</td>
<td>50</td>
<td>9o</td>
<td>53</td>
</tr>
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</table>
HCV drug screening and, therefore, has been considered adequate to evaluate anti-HCV potential candidates. The anti-HCV potential of peptide mimetics derived from isomannide 9a–o was evaluated by using the HCV replicon-based assay. The values of IC_{50} and CC_{50} of these compounds were determined (Table 2) and compounds 9a, 9g, and 9h demonstrated anti-HCV activity with IC_{50} values of 75, 35, and 100 μM, respectively. In terms of structure–activity relationship it can be seen that the activity of the thiophene derivative must be derived from an interaction of this residue with cysteine residues that are present in the active site of the enzyme. Computational studies are in progress to confirm this hypothesis.

The search for new classes of anti-HCV drugs has become imperative if we consider the numerous reports on the loss of HCV-specific reactivity was related to the antiviral effect of the compound rather than cytotoxicity. Further investigations are required to evaluate whether in vitro reduction in viral replication will be confirmed by protease-catalyzed enzyme assay.

All solvents were purchased as reagent grade, dried, using standard conditions, and stored over molecular sieves. Purification of products was carried out using silica gel flash chromatography (Whatman 60, 230–400 mesh). Routine NMR analyses were carried out on a Varian Unity Plus-300 spectrometer. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. HRMS were performed on a Waters Micromass Q-ToF Micro mass spectrometer equipped with a lock spray source. The IR spectra were obtained on a Perkin-Elmer spectrometer model Spectrum One in liquid film and KBr pellets. Optical rotations were performed on a Perkin-Elmer 341 LC polarimeter.

**Table 2** Activity of Different Peptide Mimetic Inhibitors in the HCV Luc/Ubi/Neo Replicon

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC_{50} (μM)</th>
<th>IC_{50} (μM)</th>
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</thead>
<tbody>
<tr>
<td>thiophene</td>
<td>&gt;100</td>
<td>5</td>
</tr>
<tr>
<td>9a</td>
<td>&gt;100</td>
<td>75</td>
</tr>
<tr>
<td>9b</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>9c</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>9d</td>
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<td>9e</td>
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<tr>
<td>9f</td>
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<td>ns</td>
</tr>
<tr>
<td>9g</td>
<td>&gt;100</td>
<td>35</td>
</tr>
<tr>
<td>9h</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>9i</td>
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<tr>
<td>9j</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>9k</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>9l</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>9m</td>
<td>nt</td>
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</tr>
<tr>
<td>9n</td>
<td>nt</td>
<td>nt</td>
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<tr>
<td>9o</td>
<td>nt</td>
<td>nt</td>
</tr>
</tbody>
</table>

* Measured in Huh7 cells.

<table>
<thead>
<tr>
<th>Activity</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9a</td>
<td>75</td>
</tr>
<tr>
<td>9g</td>
<td>35</td>
</tr>
<tr>
<td>9h</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

The search for new classes of anti-HCV drugs has become imperative if we consider the numerous reports on the long-term toxicity to the host and the acquired resistance to the currently available drugs for anti-HCV therapy.34,35

In this work we present the synthesis of a series of peptide mimetic compounds 9a–o and their anti-HCV potential evaluated in vitro using HCV subgenomic replicon system. Three compounds (9a, 9g, and 9h) demonstrated anti-HCV activity with IC_{50} values of 75, 35, and 100 μM, respectively. At these concentrations compounds 9a, 9g, and 9h, the cell viability was not altered suggesting that...
13C NMR (75 MHz, CDCl3): δ = 137.4, 128.3, 127.8, 127.4, 126.9, 86.9, 79.8, 78.9, 72.5, 71.2, 69.6, 57.4.


1,4,3,6-Dianhydro-2-[[Z]-2-(benzoylamo)-3-(4-chlorophenyl)propenoyl]amino]-5-O-benzyl-2-deoxy-d-glucitol (9c)
White solid; yield: 60%; mp 203–204 °C.

[a]D20 +65 (c 0.10, DMSO).

IR (KBr): 3400, 3242, 3065, 2938, 2864, 1614, 1518, 1479, 1385, 1319, 1272, 1099, 1075, 910, 754, 698 cm–1.

1H NMR (300 MHz, DMSO-d6): δ = 7.85–7.80 (m, 7 H), 7.04 (s, 1 H), 4.87–4.83 (m, 2 H), 4.48 (d, J = 11.7 Hz, 2 H), 4.23 (br s, 1 H), 4.02 (dd, J = 6.9, 4.8 Hz, 1 H), 3.98 (dd, J = 5.4, 3.9 Hz, 1 H), 3.85–3.80 (m, 2 H), 3.56 (t, J = 7.8 Hz, 1 H).

13C NMR (75 MHz, DMSO-d6): δ = 165.7, 165.3, 138.2, 133.4, 131.6, 131.0, 130.9, 128.2, 128.1, 127.7, 127.5, 127.4, 126.2, 121.4, 86.7, 79.8, 78.9, 72.4, 71.1, 69.5, 57.3.


1,4,3,6-Dianhydro-2-[[Z]-2-(benzoylamo)-3-[4-(trifluoromethyl)phenyl]propenoyl]amino]-5-O-benzyl-2-deoxy-d-glucitol (9f)
White solid; yield: 50%; mp 183–184 °C.

[a]D20 +23 (c 0.10, DMSO).

IR (KBr): 3390, 3254, 3069, 2934, 2868, 1614, 1518, 1479, 1324, 1277, 1169, 1121, 1068, 1014, 910, 747, 699 cm–1.

1H NMR (300 MHz, DMSO-d6): δ = 10.0 (s, 1 H), 8.50 (d, J = 6.6 Hz, 1 H), 7.95 (d, J = 7.5 Hz, 2 H), 7.76–7.49 (m, 5 H), 7.35–7.28 (m, 7 H), 7.07 (s, 1 H), 4.67–4.63 (m, 2 H), 4.48 (d, J = 11.7 Hz, 2 H), 4.21 (br s, 1 H), 4.07 (dd, J = 6.9, 4.8 Hz, 1 H), 3.97 (dd, J = 5.7, 3.6 Hz, 1 H), 3.83 (dd, J = 6.3, 2.4 Hz, 2 H), 3.56 (t, J = 7.5 Hz, 1 H).

13C NMR (75 MHz, DMSO-d6): δ = 165.9, 165.2, 138.6, 138.2, 133.4, 132.3, 131.7, 129.6, 128.2, 128.1, 127.9, 127.5, 127.4, 125.9, 122.2, 86.7, 79.9, 78.9, 72.5, 71.2, 69.3, 57.4.

14.3.6-Dianhydro-2-\{[(Z)-2-(benzoylamino)-3-(4-methoxyphenyl)propenoyl]amino\}-5-O-benzyl-2-deoxy-D-glucitol (9f)

Yellow solid; yield: 57%; mp 167–168 °C.

[α]D0 +75 (c 0.10, DMSO).

IR (KBr): 3334, 3059, 2949, 2878, 1643, 1622, 1604, 1510, 1482, 1368, 1293, 1176, 1095, 1025, 827, 697 cm⁻¹.

1H NMR (300 MHz, DMSO-d6): δ = 9.85 (s, 1 H), 8.27 (d, J = 6.9 Hz, 1 H), 8.00 (d, J = 6.6 Hz, 2 H), 7.59–7.50 (m, 4 H), 7.36–7.29 (m, 5 H), 7.10 (s, 1 H), 6.91 (d, J = 9.0 Hz, 2 H), 4.67–4.63 (m, 2 H), 4.48 (d, J = 11.7 Hz, 2 H), 4.21 (br s, 1 H), 4.12–4.06 (m, 1 H), 3.97 (dd, J = 5.1, 4.2 Hz, 2 H), 3.85–3.78 (m, 2 H), 3.74 (s, 3 H), 3.55 (t, J = 7.5 Hz, 2 H).

13C NMR (75 MHz, DMSO-d6): δ = 165.4, 159.4, 133.2, 132.3, 131.5, 130.9, 128.6, 128.2, 128.0, 127.8, 127.1, 127.7, 127.4, 127.3, 113.8, 86.8, 79.7, 78.9, 72.5, 71.2, 69.5, 57.3, 55.0.


Pale yellow solid; yield: 50%; mp 99–100 °C.

[α]D0 +71 (c 0.10, DMSO).

IR (KBr): 3422, 3266, 3060, 2946, 2876, 1651, 1515, 1472, 1364, 1279, 1134, 1091, 1021, 928, 805, 744, 705 cm⁻¹.

1H NMR (300 MHz, DMSO-d6): δ = 9.75 (s, 1 H), 8.30 (d, J = 6.9 Hz, 1 H), 8.04 (d, J = 6.9 Hz, 2 H), 7.75 (dd, J = 1.8, 0.6 Hz, 1 H), 7.63–7.50 (m, 3 H), 7.35–7.28 (m, 5 H), 7.04 (s, 1 H), 6.68 (d, J = 3.6 Hz, 1 H), 6.57 (d, J = 3.0, 1.8 Hz, 1 H), 4.66–4.61 (m, 2 H), 4.52 (d, J = 11.7 Hz, 2 H), 4.22 (br s, 1 H), 4.11–4.04 (m, 1 H), 4.01–3.95 (m, 1 H), 3.84–3.77 (m, 2 H), 3.55 (dd, J = 8.7, 7.5 Hz, 1 H).

13C NMR (75 MHz, DMSO-d6): δ = 165.4, 164.6, 149.6, 144.2, 138.1, 133.7, 131.5, 128.2, 128.1, 127.7, 127.4, 127.3, 116.6, 112.0, 86.6, 79.7, 78.9, 72.4, 71.1, 69.5, 57.3.


2-\{[(Z)-2-(Acetamino)-3-(2-thienyl)propenoyl]amino\}-1,4-dianhydro-5-O-benzyl-2-deoxy-D-glucitol (9k)

White yellow solid; yield: 50%; mp 78–80 °C.

[α]D0 +85 (c 0.10, DMSO).

IR (KBr): 3249, 3066, 2945, 2875, 1655, 1616, 1526, 1423, 1367, 1269, 1209, 1131, 1088, 1052, 912, 853, 737, 703 cm⁻¹.

1H NMR (300 MHz, DMSO-d6): δ = 9.22 (s, 1 H), 8.10 (d, J = 6.9 Hz, 1 H), 7.71 (d, J = 5.1 Hz, 1 H), 7.49 (s, 1 H), 7.42–7.29 (m, 2 H), 7.14 (dd, J = 5.4, 3.6 Hz, 1 H), 4.69–4.64 (m, 2 H), 4.54–4.46 (m, 1 H), 4.20 (br s, 1 H), 4.11 (q, J = 7.2 Hz, 1 H), 4.01–3.96 (m, 1 H), 3.86–3.77 (m, 2 H), 3.59–3.54 (m, 1 H), 2.05 (s, 3 H).

13C NMR (75 MHz, DMSO-d6): δ = 163.9, 164.8, 138.3, 136.7, 131.7, 129.7, 128.1, 127.5, 127.4, 126.9, 124.1, 86.7, 79.5, 78.7, 72.2, 70.9, 69.2, 57.0, 22.7.


14.3.6-Dianhydro-2-\{[(Z)-2-(benzoylamino)-3-(3-pyridyl)propenoyl]amino\}-5-O-benzyl-2-deoxy-D-glucitol (9l)

White solid; yield: 40%; mp 180–182 °C.

[α]D0 +64 (c 0.10, DMSO).

IR (KBr): 3429, 3267, 3059, 2948, 1651, 1621, 1538, 1471, 1331, 1277, 1140, 1093, 1045, 994, 893, 752, 698 cm⁻¹.

1H NMR (300 MHz, DMSO-d6): δ = 9.94 (s, 1 H), 8.46 (d, J = 6.6 Hz, 1 H), 8.11 (d, J = 6.9 Hz, 2 H), 7.87 (dd, J = 7.5, 5.1 Hz, 2 H), 7.85 (s, 1 H), 7.75–7.57 (m, 4 H), 7.34–7.28 (m, 6 H), 4.67–4.63 (m, 2 H), 4.52 (d, J = 11.4 Hz, 2 H), 4.24 (br s, 1 H), 4.13–4.06 (m, 1 H).
H), 4.03–3.96 (m, 1 H), 3.85–3.80 (m, 2 H), 3.56 (dd, J = 8.7, 7.5 Hz, 1 H).

\(^1\)C NMR (75 MHz, DMSO-\(\text{d}_6\)); \(\delta = 166.2, 164.6, 140.4, 138.2, 138.1, 137.1, 133.6, 131.8, 129.4, 128.6, 128.4, 128.2, 128.0, 127.6, 127.5, 125.6, 124.8, 124.7, 124.0, 122.2, 86.8, 79.8, 78.9, 72.5, 71.2, 69.6, 57.5.

HRMS (FAB); \(m/z [M + H]^+\) calcd for C\(_{31}\)H\(_{29}\)N\(_2\)O\(_6\): 538.2342; found: 538.2360.

1,4,3,6-Dianhydro-2-[(Z)-2-(benzoylamino)-3-(2-naphthyl)propenoyl]amino]-5-O-benzyl-2-deoxy-D-glucitol (9n)

White solid; yield: 53%; mp 152–153 °C.

\([\text{rl}]\)\(^{19}\)F 69 ± 0.10, DMSO.

IR (KBr): 3058, 2944, 2876, 1639, 1516, 1478, 1280, 1154, 1145, 1133, 1102, 906, 744, 704 cm\(^{-1}\).

HRMS (FAB); \(m/z [M + Na]^+\) calcd for C\(_{33}\)H\(_{32}\)N\(_3\)O\(_5\): 557.2025; found: 557.2070.

1,4,3,6-Dianhydro-2-[(Z)-2-(benzoylamino)-3-(1H-indol-3-yl)propenoyl]amino]-5-O-benzyl-2-deoxy-D-glucitol (9o)

Pale yellow solid; yield: 55%; mp 177–178 °C.

\([\text{rl}]\)\(^{19}\)F 59 ± 0.10, DMSO.

IR (KBr): 3252, 3058, 2946, 2877, 1716, 1645, 1531, 1479, 1454, 1378, 1330, 1284, 1218, 1136, 1016, 937, 750, 700 cm\(^{-1}\).

\(^1\)H NMR (300 MHz, DMSO-\(\text{d}_6\)); \(\delta = 9.88 \) (s, 1 H), 8.46 (d, J = 6.9 Hz, 1 H), 8.31 (d, J = 7.5 Hz, 1 H), 8.03 (d, J = 6.6 Hz, 3 H), 7.84 (d, J = 7.2 Hz, 1 H), 7.59–7.49 (m, 4 H), 7.38–7.30 (m, 8 H), 4.69–4.65 (m, 2 H), 4.54–4.50 (m, 2 H), 4.27 (br s, 1 H), 4.15–4.08 (m, 1 H), 4.04–3.99 (m, 1 H), 3.86–3.82 (m, 2 H), 3.58 (dd, J = 8.4, 7.5 Hz, 1 H).

\(^1\)C NMR (75 MHz, DMSO-\(\text{d}_6\)); \(\delta = 169.8, 164.9, 138.2, 134.4, 133.6, 136.1, 130.3, 129.2, 128.2, 128.1, 127.7, 127.5, 127.4, 126.6, 125.2, 123.6, 119.2, 118.6, 115.8, 114.7, 86.8, 79.8, 78.9, 72.5, 71.2, 69.6, 57.4.

HRMS (FAB); \(m/z [M + H]^+\) calcd for C\(_{32}\)H\(_{30}\)N\(_2\)O\(_6\): 538.2342; found: 538.2360.

HCV Replicon

The HCV subgenomic replicon named I389/3-3’-LucUbiNeo, a gift from R. Bartenschlager, was previously described. Briefly, the replicon codes for HCV NS3 through NS5B nonstructural genes under the ecephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and neomycin resistance under the HCV IRES. The Luc reporter is used as an indirect measurement of HCV replication and the activity of the Luc reporter is proportionally related to HCV RNA levels.

Cell Culture

Huh-7 cells (Hepatoma cell line) were grown at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Huh-7 cells were transfected with RNA transcribed from linearized pHCVreplicon plasmid (Ribomax Large Scale RNA production-Promega). Transient transfections were performed as previously described. Transfected cells were selected and grown in G418 (Geneticin; Gibco) at 500 μg/mL, which was absent in all experiments. Cell growth was monitored by counting the number of viable cells with trypan blue staining. Cells were treated with the recombinant human IFN (100 IU/mL in DMEM) for 18 h at 37 °C, as positive control, unless stated otherwise.

IC\(_{50}\) and CC\(_{50}\) Determinations Using the Replicon System

Briefly, 5,000 Huh-7 cells containing HCV subgenomic replicon were plated in 96-well plates in a total volume of 100 μL of growth medium in Dulbecco’s modified Eagle medium containing 5% (vol/vol) fetal bovine serum without G418. Inhibitors were added 24 h post-plating in threefold dilutions at a final DMSO concentration of 1% (vol/vol). After 3 d, cells were harvested and the firefly luciferase signal was observed compared to untreated cells. Human interferon alpha (IFN-α) and thiophene was included in each run as a positive control. Subconfluent cultures of the Huh.7 cell line were plated out into 96-well plates and used for analysis of cell viability (cytotoxicity) or antiviral activity. The cytotoxicity of each compound was assessed as a percent of viable cells relative to untreated cells using CellTiter-Blue (Promega), a colorimetric assay used as an indicator for cell viability.

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