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Novel inhibitors of the hepatitis C virus NS3 proteinase

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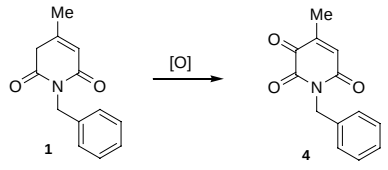
Abstract—The hepatitis C virus proteinase inhibitor 4-methyl-1-(phenylmethyl)-2,6-pyridinedione **1** undergoes a novel autoxidation process, on silica gel, leading to the dimer **2** as the major product, a relatively more potent inhibitor of the enzyme.

Hepatitis C is the identified etiologic agent of parentally transmitted non-A, non-B hepatitis (NANBH). Currently, approximately 170 million individuals worldwide have developed chronic hepatitis C and if left untreated may progress to liver cirrhosis or hepatocellular carcinoma.[1–4] Originally, the only effective therapeutic agent was the prolonged use of interferon-α (IFN-α); however, a large proportion of patients (>50%) subsequently relapse once the drug is withdrawn. More recently, the introduction of the combination of pegylated IFN and ribavirin improves efficacy in patients infected with HCV genotype 2 or 3 to approximately 80%; however, the response rate of patients infected with genotype 1 is still less than 50%.[5] This clearly indicates a necessity to identify new therapeutic targets and develop small molecule therapeutic agents that are not only antiviral agents in their own right but can also improve the effectiveness of immunotherapeutic agents such as IFN-α. One such approach involves the development of molecules that inhibit the virus encoded NS3 serine protease. The hepatitis C virus 9500 nucleotide RNA genome encodes a 3000 amino acid polyprotein that is proteolytically processed into at least 10 products.[6,7] The NS3 proteinase is responsible for processing four of these cleavage sites believed to be essential for virus replication, thus making the enzyme an attractive antiviral target.[8,9]

While searching for new HCV proteinase inhibitors, we discovered that 4-methyl-1-(phenylmethyl)-2,6-pyridinedione **1** exhibited modest activity in a cell-free translation assay (IC_{50} = 47 μM).[10] In an attempt to find more potent derivatives, we envisioned that the trione **4** (Scheme 1) possibly obtained from oxidizing the dione **1**, would be a particularly attractive target since α-ketoamides and other related 1,2-diketo compounds are well-known inhibitors of serine proteases[11] including the NS3 protease.[12] During the purification and routine handling of the imide **1**, interesting color changes on silica gel took place. We reasoned that an autoxidation reaction was possibly occurring, hopefully leading to the trione **4**, and hence, we decided to investigate these observations further by adsorbing this compound on silica gel and leaving it exposed to the atmosphere. After approximately 0.5 h, the silica gel turned dark blue and then to brown when left overnight (16 h). After repeating this process twice, the products were obtained and purified by passage through a silica gel column providing the 'dimers' **2** and **3** in 59% and 5% yields, respectively (Scheme 2).[13] Almost three decades ago, Nasipuri and co-workers observed that 3,4-dihydro-1-methyl-naphthalen-2(1*H*)-one **5**, an oil, produced a crystalline substance, the 'dimer' **6** (10–15% yield), when left exposed to the atmosphere.[14] Also generated from the same



Scheme 1.

Keywords: Hepatitis; Proteinase.
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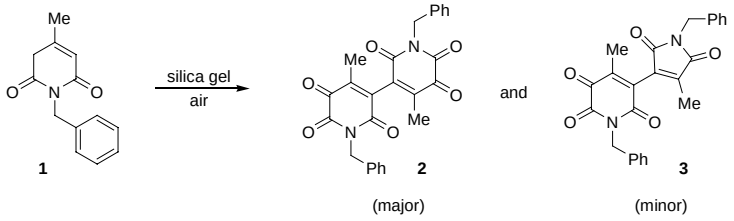


Scheme 2.

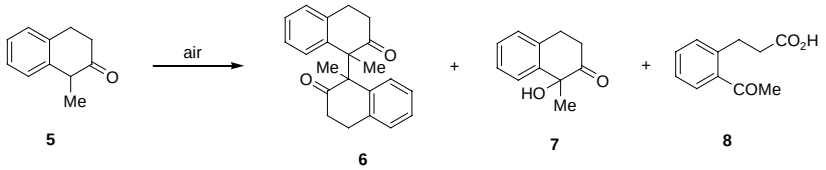
experiment were the 'expected' products characteristic of aerial oxidation of related cyclic ketones, the α-hydroxy ketone **7** (2–5%) and the acid **8** (30%), both derived from the corresponding α-hydroperoxy ketone (Scheme 3).[15]

In the present example, it is possible that an analogous process takes place, via the radical **9** to the adduct **10** which could not be isolated because it contains keto groups that form stable enolates and hence vulnerable to further oxidation. Dehydration of the intermediate hydroperoxides leads to the major product **2** (Scheme 4; Path A). The minor product **3** probably results from the same peroxides partially by dehydration and ring cleavage to the unstable diacid **11**. Decarboxylation and subsequent cyclization provide the five-membered ring product **3** (Path B). Most interestingly, the dimer **2** proved to be a potent inhibitor in the cell-free translation assay (IC_{50} = 6 μM). Furthermore, activity was confirmed in an enzyme assay (IC_{50} = 0.083 μM).[16]

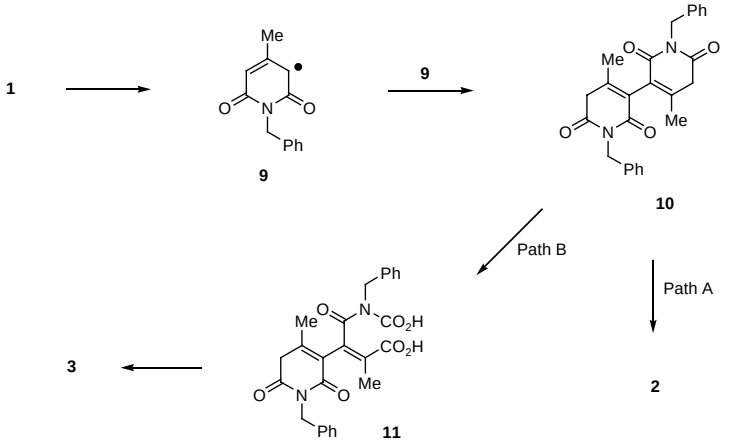
The desired product **4** was eventually obtained in 21% yield by adding the starting material **1** to a stirred suspension of Dess–Martin periodinane (1.5 eqiuv) in dichloromethane.[17] Although more potent than its precursor **1** (IC_{50} = 4.0 μM; enzyme assay), the trione **4** proved to be relatively a less active inhibitor (IC_{50} = 0.44 μM; enzyme assay) than the 'dimer' **2** and hence of less immediate interest.

In an attempt to initiate an understanding of the role of our lead compound **2** in the inhibition of HCV proteinase, an analysis was carried out in a continuous spectrophotometric assay.[18] The data were applied to a non-linear curve using the equation $P = v_s t + (v_o - v_s)(1 - e^{-kt})/k$. With the initial velocity v_o, final steady-state velocity, v_s, and the apparent first-order rate constant k, the K_i, and K_i^* constants were calculated to be 40 and 9 μM, respectively. Furthermore, the on and off rates, k_5 and k_6, were determined to be 0.093 and 0.028 min^{-1}. Since the rates are of a magnitude which was observable during the attainment of equilibrium, the fact that the K_i^* is smaller than the K_i conforms to a mechanism of slow-binding inhibition. The isomerization constant (k_5/k_6) is 3, and since the off rate is equal to 0.028 min^{-1}, we can conclude that the dimer 2 is a reversible inhibitor.

In summary, a potent HCV protease inhibitor, the symmetrical dimer **2**, is obtained from a novel autoxidation reaction involving the monomeric imide **1** on silica gel.



Scheme 3.



Scheme 4.

In a cell-free translation assay for the HCV NS3 protease, the oxidized dimer **2** ($IC_{50} = 6\ \mu M$) proved to be considerably more potent inhibitor than the monomer **1** ($IC_{50} = 47\ \mu M$), representing an approximate 8-fold increase in potency. Subsequently, using a continuous spectrophotometric assay, it was determined that this compound inhibits the enzyme reversibly.

Extensions of this study, including selectivity toward other serine proteinases of the compounds (**1**, **2**, and **4**) described in this article on the enzyme, will be published elsewhere.

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10. Cell-free translation assay for HCV NS3-protease-translated substrate was prepared as follows: The plasmid pNBNAE was linearized with *Eco*RI and transcribed with T7 RNA polymerase (Promega) to produce RNA-encoding HCV polyprotein ΔNS4A/Δ4B from amino acid residue 1693 to 1903 in reticulocyte lysates (Promega) in the presence of [^{35}S]methionine. Translation reactions were terminated by adding DNAse-free RNAse (Boehringer–Mannheim) and cyclohexamide (Sigma) to 10 μg/ml followed by incubation at 30 °C for 15 min.Protease assays were initiated by the addition of 2.5 nM 95% pure 631/4A HCV protease to 2 μl ^{35}S-labeled translated substrate in a 20 μl volume containing 10 mM Tris, pH 7.5, 120 mM NaCl, 5 mM DTT, 0.5% EDTA, 0.1% Tween 20, and 12% glycerol followed by incubation at 30 °C for 30 min. Inhibitors were added to assay mixtures prior to incubation. In-house standards of HCV protease inhibitor molecules served as positive controls. Cleavage reactions were terminated by addition of an equal volume of 2× Laemmli sample buffer and boiling for 3 min. Cleavage products were analyzed by SDS/15% PAGE gel electrophoresis and autoradiography.
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13. *Procedure*. Silica gel (Merck grade 60, 230–240 mesh, 60 A; 1.00 g) was added to a dichloromethane (5 ml) solution of the dione **1** (0.100 g; 0.47 mmol) and the resulting suspension was concentrated under reduced pressure. The resulting solid was left at room temperature, exposed to the atmosphere, for 16 h, and then washed thoroughly with dichloromethane, filtered, and concentrated. This protocol was repeated twice and the final residue was applied to a silica gel column and eluted with ethyl acetate–hexane (4:6) to give (i) the ring-contracted product **3** (0.005 g; 5%) as a yellow solid, δ_H (200 MHz; $CDCl_3$): 1.98 (3H, s), 2.00 (3H, s), 4.70 (2H, s), 5.09 (2H, ABq), and 7.22–7.50 (10H, m). FAB-MS: MH^+, 429, followed by (ii), the symmetrical dimer 2 (0.063 g; 59%) again as a yellow solid, δ_H (200 MHz; $CDCl_3$): 1.96 (6H, s), 5.09 (4H, ABq), and 7.30–7.41 (10H, m); δ_c ($CDCl_3$): 174.99, 160.74, 154.66, 145.00, 137.44, 135.06, 129.22, 128.71, 128.72, 44.39, and 13.54. HRFAB-MS: MH^+, 457.1407. $C_{26}H_{20}N_2O_6$ requires 457.1400.
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16. Inhibition of proteolytic activity of HCV NS3 protease determined by scintillation proximity assay (SPA). In this assay, the purified NS3 631/4A protease was preincubated with the inhibitor for 1 h at 4 °C. Reactions were initiated by adding the NS5A/5B peptide substrate Biotin-Asp-Thr-Glu-Asp-Val-Val-Cys-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-

Gly-Lys (3H), and then incubated for 2 h at room temperature (21 °C). Final reaction conditions were: 50 mM MOPS, pH 7.5, 0.3 M NaCl, 0.1% NP40, 0.5 mM DTT, 20% glycerol, 100 μg/ml BSA, 10 nM HCV protease, and 2 μM substrate peptide. $ZnCl_2$ and in-house standards of HCV protease inhibitor molecules served as positive controls. After stopping the reaction, cleavage of the Cys–Ser bond was detected by the addition of streptavidin-coated SPA beads (Amersham), which can bind to the biotin-labeled peptide. Cleavage is indicated by reduction in signal caused by separation of the radiolabeled fragment from the bound biotinylated fragment (measured in CPM) which is proportional to proteolytic activity.

17. *Procedure*. The dione **1** (0.430 g; 2.0 mmol) in dichloromethane (5 ml) was added, in one portion, to a suspension of the Periodinane (1.27 g; 3.0 mmol) in dichloromethane (5 ml) while cooled at 0 °C. The resulting mixture was removed from the ice bath and stirred for a further 2 h. The reaction was partitioned between dichloromethane and aq 10% $Na_2S_2O_3$. The organic phase was separated, washed with aq $NaHCO_3$, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using ethyl acetate–hexane (1:3) as eluent to provide the trione **4** (21%) as a yellow solid, δ_H (200 MHz; $CDCl_3$): 2.15 (3H, d), 5.09 (2H, s), 6.91 (1H, m) and 7.49–7.31 (5H, m); δ_c ($CDCl_3$): 165.22, 162.04, 151.82, 146.77, 135.63, 134.02, 129.44, 128.69, 128.18, 44.90, and 15.23. HRFAB-MS: MH^+, 230.0817. $C_{13}H_{11}NO_3$ requires 230.0817.
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