



## SMALL PEPTIDIC ALDEHYDE INHIBITORS OF HUMAN RHINOVIRUS 3C PROTEASE

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**Abstract:** Small peptide aldehydes were designed to mimic the preferred substrate requirements for the human rhinovirus 3C protease. Di- and tripeptide aldehydes containing a methionine sulfone as a P<sub>1</sub> surrogate for glutamine show low micromolar enzyme inhibitory and antiviral tissue culture activity. LY338387, obtained in a short and efficient synthesis, appears to validate the protease as a therapeutic target.

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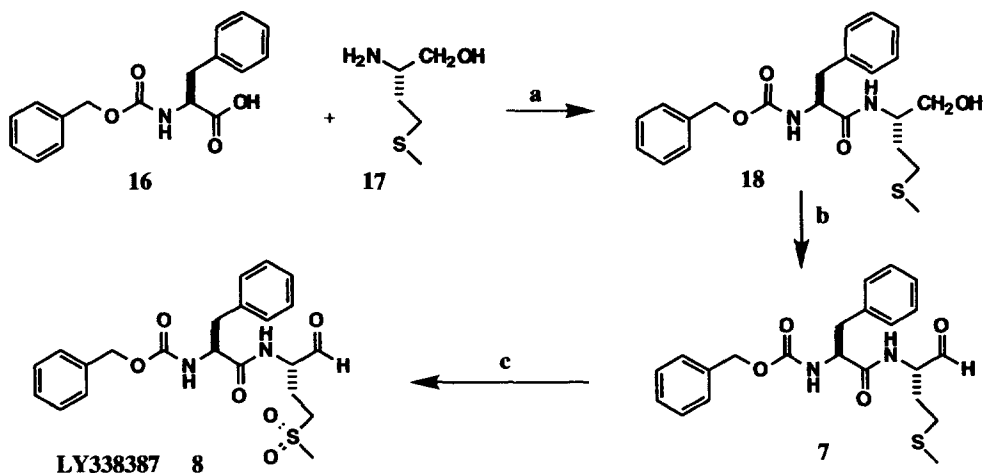
The human rhinoviruses (HRVs) are believed to be the major cause of the common cold.<sup>1</sup> Their positive strand RNA genome is translated directly into a polyprotein that is cleaved by the 3C proteinase (3C<sup>pro</sup>), or its precursor 3CD, resulting in mature capsid and viral proteins that ultimately assemble into infectious virions.<sup>2</sup> The 3C<sup>pro</sup> was recently shown to be a cysteine protease in a trypsin-like fold.<sup>3</sup> Because 3C<sup>pro</sup> makes highly specific cleavages that are essential to viral replication, it would appear to be an attractive target for antiviral therapy.

3C<sup>pro</sup> makes its cleavages primarily at the Gln-/Gly bond. Colonna and coworkers determined the hexapeptide Thr-Leu-Phe-Gln-/Gly-Pro to be the minimally effective substrate at the 2C-3A cleavage site.<sup>4</sup> Relatively few inhibitors of HRV 3C<sup>pro</sup> have been reported and none have demonstrated low micromolar antiviral activity.<sup>5-8</sup> Recently two tetrapeptide aldehydes, based upon the above sequence requirement (P<sub>4</sub>-P<sub>1</sub>), were reported to be good enzyme inhibitors.<sup>9,10</sup> However, the combination of the P<sub>1</sub> glutamine and aldehyde functionalities presents an interesting dilemma. In the case of the Kaldor et al. inhibitor **15** (N-Boc-Leu-Val-Phe-Gln-CHO) the free glutamine cyclizes on the aldehyde to provide the preferred glutaminal tautomer.<sup>10</sup> The compound retains good enzyme inhibition (IC<sub>50</sub> = 0.6 μM) but lacks good tissue culture activity (IC<sub>50</sub> = 400 μM).<sup>11</sup> The Malcolm group tetrapeptide (N-Ac-Ala-Ala-Ala-Gln (NMe<sub>2</sub>)-CHO) protects the glutamine as the dimethyl amide and also retains good enzyme inhibition even though the sequence is obviously not optimized for HRV 3C<sup>pro</sup> (K<sub>i</sub> = 0.34 μM).<sup>9</sup> These results were consistent with their hypothesis that "an uncharged δ-carbonyl oxygen is required in the P<sub>1</sub> position of the substrate for efficient recognition by the picornaviral enzymes." Unfortunately, no antiviral data was reported for this inhibitor.

We thought that a P<sub>1</sub> Met (SO<sub>2</sub>) might effectively mimic the glutamine and simplify the above synthetic limitations. Compound **2** (Table 1) is a commercially available tripeptide calpain II inhibitor that we imagined could test this isostere concept by a simple one-step oxidation of the methionine. We were encouraged that this compound **3** (LY335230) showed good enzyme inhibition (K<sub>i</sub> = 0.49 μM),<sup>12</sup> potent in vitro translation inhibition,<sup>13,14</sup> and apparent antiviral activity (Table 1).<sup>15</sup> However, to improve activity we sought to more closely mimic the substrate requirements of HRV-14 3C<sup>pro</sup>. Tsujinaka and coworkers reported the improved cell penetration for the Z-protected dipeptide calpain inhibitor calpeptin (**4**).<sup>16</sup> Others have also noted Z-protected dipeptide aldehydes to be good inhibitors of cathepsin L<sup>17</sup> and HIV protease.<sup>18</sup> These observations led us to synthesize the dipeptide LY338387 (**8**) incorporating a P<sub>2</sub> Phe substituent (Scheme 1).

In an efficient, straightforward synthesis Cbz-protected phenylalanine was coupled to L-methioninol in 55% yield. The alcohol was selectively oxidized under nonracemizing conditions to the aldehyde **7** with sulfur trioxide pyridine complex (10 min, room temperature). The methionine was then oxidized to the sulfone using Oxone®.<sup>19</sup> LY338387 was a potent reversible inhibitor of 3CP<sup>ro</sup> with a  $K_i = 0.47 \mu\text{M}$ . We also observed good tissue culture activity (Table 1,  $\text{IC}_{50} = 3.4 \mu\text{M}$ ) without cytotoxicity ( $\text{TC}_{50} > 224 \mu\text{M}$ ). Further evaluation of **3** and **8**, including an in vitro translation assay, will be reported in due course.<sup>14</sup> The precursor sulfide **7** also showed good antiviral activity but with a poorer therapeutic index (TI) (see Table 1 footnote c). We cannot rule out the possibility that some in situ oxidation of the methionine is giving rise to activities similar to the methionine sulfone analogs.

#### SCHEME 1. Synthesis of LY338387



(a) CDI, THF, 55%; (b) Pyridine-SO<sub>3</sub>, Et<sub>3</sub>N, DMSO, 94%; (c) Oxone®, MeOH, H<sub>2</sub>O, 85%.

A short SAR was then initiated to examine the effect of changing the P<sub>2</sub> Phe substituent. Compounds **6**, **10**, **12**, and **13** were synthesized in the same general manner as shown in Scheme 1. Substitution of Leu for Phe (**6**) caused a 12-fold loss in antiviral activity. Substitution of homophenylalanine for Phe (**12**) caused a 24-fold loss in antiviral activity while the D-Phe analog (**13**) was completely devoid of antiviral activity.

Replacement of the Cbz protecting group of LY338387 with the Boc group resulted in a weak enzyme inhibitor (**10**,  $\text{IC}_{50} = 60 \mu\text{M}$ ) and a 21-fold loss in antiviral activity. Finally, we wanted to make the Z-protected dipeptide analog in the glutamine aldehyde series to see if the antiviral activity would be improved. Kaldor et al. reported that the Boc protected dipeptide (N-Boc-Phe-Gln-CHO) was a poor enzyme inhibitor ( $\text{IC}_{50} > 80 \mu\text{M}$ ).<sup>10</sup> We followed their reported procedure for making the glutarimide of glutamine, which was coupled to N-Cbz-Phe, followed by selective reduction of one of the glutarimide carbonyls with sodium borohydride to give compound **14**. We were pleased to find an almost 10-fold improvement in antiviral activity over the tetrapeptide **15** even though the compound was less potent as an enzyme inhibitor ( $\text{IC}_{75} = 60 \mu\text{M}$ ).

In summary, we are the first to report dipeptide aldehydes which give low micromolar antirhinoviral (HRV-14) activity in tissue culture. LY338387 can be rapidly synthesized and appears to be a suitable positive control for a high throughput 3C protease inhibitor assay.<sup>20</sup> These results suggest the 3C protease could be a useful therapeutic target for antiviral therapy.

**TABLE 1. Antiviral Activities of Peptide Aldehydes**

Compound	IC <sub>50</sub> (μM) <sup>a</sup>	TC <sub>50</sub> (μM) <sup>b,c</sup>
1 N-Ac-Leu-Leu-Arg-CHO (Leupeptin)	>224	>224
2 N-Ac-Leu-Leu-Met-CHO	23.7	37.1
3 N-Ac-Leu-Leu-Met (SO <sub>2</sub> )-CHO (LY335230)	81.9	>224
4 N-Cbz-Leu-Nle-CHO (Calpeptin)	17.7	22.1
5 N-Cbz-Leu-Met-CHO	46.8	83.0
6 N-Cbz-Leu-Met (SO <sub>2</sub> )-CHO	39.3	>224
7 N-Cbz-Phe-Met-CHO	9.7	42.2
8 N-Cbz-Phe-Met (SO <sub>2</sub> )-CHO (LY338387)	3.4	>224
9 N-Boc-Phe-Met-CHO	84.1	65.4
10 N-Boc-Phe-Met (SO <sub>2</sub> )-CHO	71.3	147.6
11 N-Cbz-HPhe-Met-CHO <sup>d</sup>	20.3	13.5
12 N-Cbz-HPhe-Met (SO <sub>2</sub> )-CHO	81.2	161.5
13 N-Cbz-D-Phe-Met (SO <sub>2</sub> )-CHO	>224	>224
14 N-Cbz-Phe-Gln-CHO	42.0	>224
15 N-Boc-Val-Leu-Phe-Gln-CHO	400 <sup>(11)</sup>	

<sup>a</sup>Antiviral activity measured by 48 h plaque reduction assay (HRV-14, HeLa cells).<sup>15</sup> <sup>b</sup>Cytotoxic effect measured by XTT assay.<sup>13</sup> <sup>c</sup>TI, therapeutic index (defined as TC<sub>50</sub>/IC<sub>50</sub>). By convention, true antiviral activity requires a TI ≥ 10. <sup>d</sup>HPhe = Homophenylalanine.

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11. Reference 10 gives the tissue culture  $IC_{50}$  of compound **15** as 500  $\mu$ M in the reference and notes section, whereas reference 13 gives an  $IC_{50}$  = 300  $\mu$ M. We have used an average of these reports.
12. Inhibition of purified 3C protease (Birch, G. M.; Black, T.; Malcolm, S. K.; Lai, M. T.; Zimmerman, R. E.; Jaskunas, S. R. *Protein Expr. Purif.* **1995**, *6*, 609) measured with a fluorescence assay using the substrate Anthranil-TLFQGPV(pNO<sub>2</sub>Phe)K.<sup>14</sup>
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15. Plaque reduction assay for HRV14: Confluent monolayers of HeLa cells were infected using 100 pfu per plate. After an adsorption period of 60 min the inocula were replaced by 1.5 mL of a maintenance medium overlay containing 0.5% agarose and supplemented with various concentrations of test compound. The plates were incubated at 34 °C for 48 h and the infected monolayers were then fixed with buffered 10% formalin in H<sub>2</sub>O and then stained with crystal violet after removal of the overlay. The mean plaque number was calculated from a duplicate series of counts, converted to a percentage of untreated controls and plotted against the log<sub>10</sub> concentration of test compound. This data was used to calculate the  $IC_{50}$ .
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19. All compounds gave satisfactory analytical or exact mass and spectral data. <sup>1</sup>H NMR data for LY338387: (CDCl<sub>3</sub>, 300 MHz)  $\delta$  9.31 (s, 0.5H), 7.42-7.18 (m, 10H), 7.06 (d, 1H,  $J$  = 7.3 Hz), 5.53 (d, 1H,  $J$  = 6.4 Hz), 5.05 (s, 2H), 4.45 (m, 1H), 4.32 (m, 1H), 3.10-2.91 (m, 4H), 2.83 (s, 3H), 2.40 (m, 1H), 2.02 (m, 1H). Based upon the integration of the aldehyde proton the compound appears 50% hydrated. TLC one spot  $R_f$  = 0.65 (90:10 CH<sub>2</sub>Cl<sub>2</sub>:MeOH). FABMS (M+H)<sup>+</sup> : 447. Anal. calcd for C<sub>22</sub>H<sub>26</sub>N<sub>2</sub>O<sub>6</sub>S: C, 59.18; H, 5.87; N, 6.27; Found: C, 58.98; H, 6.06; N, 6.00.
20. Steve Kahl, Rob Johnson and Q. May Wang (Lilly Research Labs) private communication.

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