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Synthesis and application of MeOSuc-Ala-Ala-Pro-Phe-CH_2Cl as potent proteinase K inhibitor

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ABSTRACT

The synthesis and proteolytic inhibitor function of two new tetrapeptides, methoxysuccinyl-Ala-Ala-Pro-Phe-chloromethyl ketone (MeOSuc-AAPF-CH_2Cl) and methoxysuccinyl-Ala-Pro-Ala-Phe-chloromethyl ketone (MeOSuc-APAF-CH_2Cl) are described. The efficacy of these two new analogs in inhibiting the proteolytic activity of proteinase K has been compared with the previously-documented proteainase K inhibitor, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSuc-AAPV-CH_2Cl). An examination of inhibitory activity using a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay in the presence of proteinase K reveals that the AAPF inhibitor (MeOSuc-AAPF-CH_2Cl) at a concentration of 0.05 mM allows a signal to be obtained for an exogenous target (“Xeno RNA”) at 30 cycles (i.e. Ct = 30), whereas the MeOSuc-AAPV-CH_2Cl control requires a 10-fold higher concentration (0.5 mM) to produce the same Ct. Interestingly, the other new analog, with the rearranged amino acid sequence APAF (MeO Suc-APAF-CH_2Cl), provides no proteinase K inhibition under the same experimental conditions. These results suggest that when P1 is phenylalanine, alanine at P2 and proline at P3 is not tolerated as a good proteinase K inhibitor. A plausible explanation for the higher efficiency of MeOSuc-AAPF-CH_2Cl over control is proposed based on the molecular modeling studies.

Active site-directed irreversible inhibitors (‘suicide inhibitors’) have been useful reagents in many enzymatic studies.[1] Specifically, with proteinase studies, this approach utilizes a synthetic peptidyl portion as a substrate analog, which directs the inhibitor to the active site of the target enzyme, attached to a mechanism specific reactive group, which then forms a covalent adduct that blocks further enzyme activity. Peptidyl chloromethyl ketones affinity labels developed for serine proteases were among the first active site-directed irreversible inhibitors reported for any enzyme.[2,3] The use of the single amino acid chloromethyl ketones, Tos-Phe-CH_2Cl (TPCK) and Tos-Lys-CH_2Cl (TLCK), as specific reagents for chymotrypsin and trypsin, respectively, is one of the classic demonstrations of the value of affinity labels for enzyme specificity studies.[4] Subsequently, halomethyl ketones were also used for the characterization of functional groups and sequences involved in active sites. They served as probes of structure by creating proteins with these specific labels to provide spectroscopic handles, fixed substrate analogs for crystallographic, and as irreversible inhibitors for the study of the biological function of specific proteases. Currently, peptide-linked halomethyl ketones are probably the most widely-used class of affinity labels.[5] These target serine proteases, which are a class of proteases involved in a number of important physiological processes including blood coagulation, the complement system, fertilization, and protein turnover. Many of these enzymes are believed to be involved in diseases such as emphysema, arthritis, and tumorogenesis. Selective regulation of these and other proteases in the disease state is an important clinical problem.[6] The proposed reaction of a serine protease with most substrate-related haloketones first involves the formation of an enzyme competitive inhibitor complex in which the inhibitor is recognized by specific interactions between the side chain of the P_1 amino acids residue and the S_1 or primary substrate-binding site of the enzyme.[7,8] This is followed by the formation of a stable covalent bond between the inhibitor and the enzyme active site. The proposed mechanism for this reaction has been deduced from the molecular structure of the chloromethyl ketone substrate with its target enzyme as determined by X-ray crystallographic studies.[14,15] These reveal that the inhibitor acts as an electrophile and the enzyme acts as a nucleophile. The overall interaction involves the formation of two covalent bonds.[14,15] First, C–N single bond formation takes places between nitrogen atom of the enzyme’s active-site histidine imidazole residue and the methylene carbon of the inhibitor. Second, C–O single bond formation takes place between the oxygen atom of the enzyme’s active-site serine hydroxyl residue and the ketone carbonyl carbon of the inhibitor. In addition to the special structural feature of chloromethyl ke-

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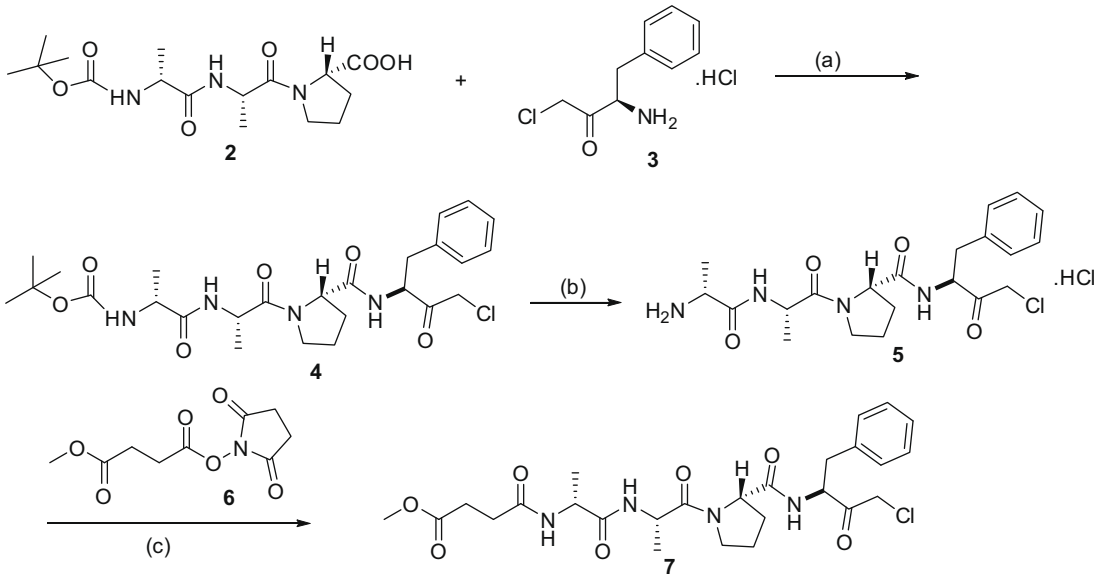
tone group, another feature is the presence of extended peptide chain that helps to increase the resemblance of the inhibitor to enzymes.

In our search efforts to find better inhibitors to eliminate the need for proteinase K removal prior to further in vitro enzymatic reactions, we set out to validate the application of newly synthesized modified tetrapeptides. The modified tetrapeptides are designed in such a way as to provide a methoxysuccinyl group at the N-terminus and a chloromethyl ketone group at the C-terminus of the tetra-amino acid chain. It appears that the presence of the methoxysuccinyl group aids binding to the recognition site of proteinase K by two hydrogen bonds, one direct between the methoxy oxygen of the succinyl group and the hydrogen of the Tyr^{104} (N), and the other mediated by a water molecule, succinyl-peptide (C + O)–Wat^{453}–(HN)Gly^{136}. We have synthesized two new proteinase K inhibitors, methoxysuccinyl-Ala-Ala-Pro-Phe-chloromethyl ketone (MeOSuc-AAPF-CH_2Cl) (Scheme 1) and methoxysuccinyl-Ala-Pro-Ala-Phe-chloromethyl ketone (MeOSuc-APAF-CH_2Cl) (Scheme 2). The known proteinase K inhibitor, MeOSuc-AAPV- CH_2Cl **1** was purchased from Bachem and used as a control. Its structure is shown in Figure 1.

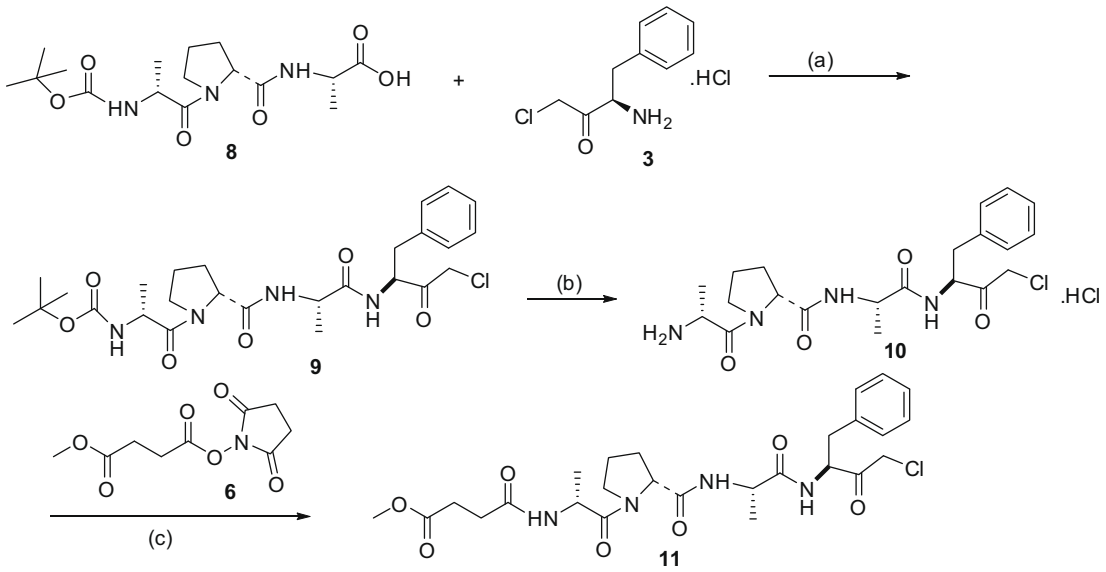
The reaction pathway leading to the formation of desired MeOSuc-AAPF-CH_2Cl **7** is depicted in Scheme 1. The peptide coupling reaction of Boc-Ala-Ala-Pro-OH **2** with H-Phe- CH_2Cl.HCl **3** via the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine using THF as the solvent afforded the corresponding Boc-AAPF-CH_2Cl **4** in 89% yield. The Boc group of **4** was cleaved using 6 M HCl/EtOAc to give the corresponding H-AAPF-CH_2Cl·HCl **5** in 88% yield. Finally, the reaction of **5** with methyl succinimido succinate **6** in the presence of THF/$NaHCO_3$ provided the corresponding MeOSuc-AAPF-CH_2Cl **7** in 70% yield.[9]

The synthetic strategy to make MeOSuc-APAF-CH_2Cl **11** was similar to the strategy developed to make MeOSuc-AAPF-CH_2Cl **7** (Scheme 2). The peptide coupling reaction of Boc-Ala-Pro-Ala-OH **8** with H-Phe-CH_2Cl.HCl **3** in the presence isobutyl chloroformate and *N*-methylmorpholine using THF as the solvent under mixed anhydride method, followed by the cleavage of the Boc group in the presence of 6 N HCl/EtOAc system afforded the corresponding H-APAF-CH_2Cl·HCl **10** in 93% yield. Treatment of **10** with methyl succinimido succinate **6** in the presence of THF/$NaHCO_3$ furnished the corresponding MeOSuc-APAF-CH_2Cl **11** in 76% yield.[10]

Reverse transcription-Polymerase Chain Reaction (RT-PCR) with real-time detection of amplification products is a robust, simple, and quantitative way to measure RNA levels in solution. These measurements can be made directly from cultured cells using the Cells-to-CT™ kit from Applied Biosystems. The biological samples receive a treatment with this process is to be digested with proteinase K, after which is it permanently inactivated by a suicide inhibitor. In order to perform the validation of the new proteinase K inhibitors **7** and **11**, we have used the TaqMan® gene expression Cells-to-CT™ kit and TaqMan® Cells-to-CT™ control kit (Applied Biosystems), using the new putative inhibitors in place of the current inhibitor, MeOSuc-AAPV-CH_2Cl **1**. The stop solutions with and without inhibitor were prepared[11,12] and reverse transcription and real-time PCR were carried out. Reverse transcription was performed using a primer specific for an exogenously added Template, Xeno RNA. The inhibitors were added at various concentrations prior to addition of the RT reaction components. The RT reaction was next incubated at 37 °C for 1 h followed by incubation at 95 °C for 5 min to deactivate the RT enzyme. The RT-reaction was next cooled to 4 °C before adding the PCR components and performing the real-time analysis using the GeneAmp® 9700 PCR system[12,13] (Applied Biosystems). The outcome of the analysis of RT and qPCR with new proteinase K inhibitors **7** and **11** were compared with the standard inhibitor **1**, and their average Ct values were plotted against the various concentrations of the other tetrapeptides (Fig. 2). As shown in Figure 2, the control proteinase K inhibitor MeOSuc-AAPV-CH_2Cl **1** has a plateau of inhibition for proteinase K from 1 to 0.5 mM concentration, resulting in a Ct of about 30, while the new inhibitor MeOSuc-AAPF-CH_2Cl **7** extends this effective range down to 0.05 mM concentration, again resulting in a Ct of about 30. Clearly, the data indicate that the compound **7** is 10-fold better proteinase K inhibitor than the control compound **1**. Meanwhile, when the other tetrapeptide MeOSuc-APAF-CH_2Cl **11,** which has proline at its P3 position and phenylalanine at P1 position, is used as the stop solution, no Xeno RNA is detected in any of the reactions (Ct = 40), indicating the proteinase K retained enough activity to eradicate any Reverse Transcriptase activity. Since it shows no inhibitory activity for proteinase K at any of the concentrations used, it clearly indicates that when P1 is phenylalanine, alanine at P2 and proline at P3 is not tolerated as a good proteinase K inhibitor.

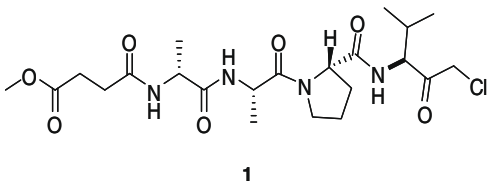


Scheme 1. Reagents and conditions: (a) isobutyl chloroformate, *N*-methylmorpholine, 4 °C, 15 h; (b) 6 N HCl/EtOAc, rt, 3 h; (c) THF/$NaHCO_3$, rt, 3 h.



Scheme 2. Reagents and conditions: (a) isobutyl chloroformate, *N*-methylmorpholine, 4 °C, 15 h; (b) 6 N HCl/EtOAc, rt, 3 h; (c)THF/$NaHCO_3$, rt, 3 h.

In order to find out why MeOSuc-AAPF-CH_2Cl **7** is a better proteinase K inhibitor than the control compound MeOSuc-AAPV-CH_2Cl **1**, we have employed computer modeling studies. The coordinates of proteinase K in complex with MeOSuc-AAPA-CH_2Cl at 2.2 Å resolution were used as a starting point for this molecular modeling study.[14,15] The program WinCoot[16] was used to model the inhibitors methoxysuccinyl-Ala^{281}-Ala^{282}-Pro^{283}-Val^{284}-CH_2Cl (MeOSuc-AAPV-CH_2Cl **1**) and methoxysuccinyl-Ala^{281}-Ala^{282}-Pro^{283}-Phe^{284}-CH_2Cl (MeOSuc-AAPF-CH_2Cl **7**) in place of MeOSuc-AAPA-CH_2Cl. Figures were created with the visualization program PyMol.[17] Figure 3 revealed that the shifts of inhibitor residue 284 were not predicted to disrupt the covalent interactions (His^{69}, Ser^{224}) and hydrogen bonding network described previously.[14,15] Examination of the active site around inhibitor residue 284 revealed a small binding pocket, shown by the surface representations in Figure 3. This binding pocket is bordered by Ala^{158}, Ala^{159}, Gly^{160}, Ser^{170}, Pro^{171}, and Ala^{172}, and potentially offers additional van der Waals, hydrogen bond, and/or hydrophobic interactions to enhance inhibitor potency.



1

Figure 1. Structure of MeOSuc-Ala-Ala-Pro-Val-CH_2Cl as a standard proteinase K inhibitor.

The observation that MeOSuc-AAPF-CH_2Cl **7** works at ten-fold lower concentration than MeOSuc-AAPV-CH_2Cl **1** is probably due the nature of chloromethyl ketone amino acid. In the case of MeOSuc-AAPF-CH_2Cl **7**, the phenylalanine has an aromatic side chain (i.e. phenyl group that could fit into the binding pocket) which makes a stronger inhibitor–enzyme complex linkage, whereas in

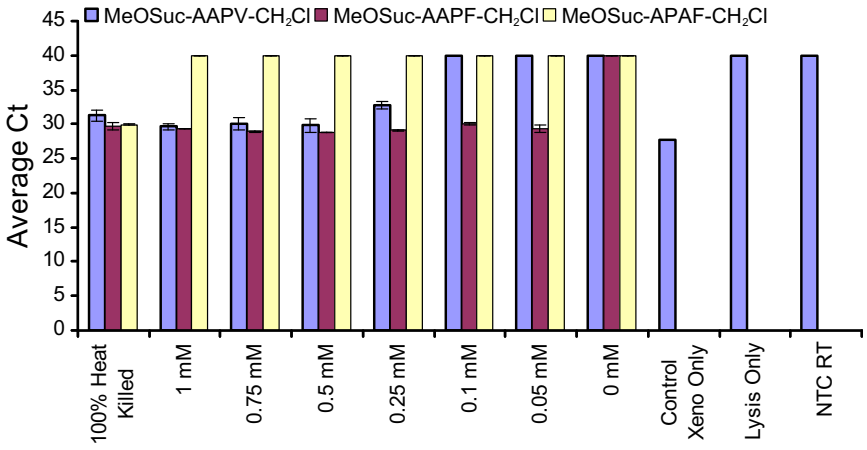


Figure 2. Histograms of average Ct (cycle threshold) values were plotted against the varying concentration of MeOSuc-AAPV-CH_2Cl **1**, MeOSuc-AAPF-CH_2Cl **7**, and MeOSuc-APAF-CH_2Cl **11**. The term 'Ct' represents the PCR cycle number when the signal is first recorded as statistically significant. Thus, the lower the Ct value, the greater the concentration of nucleic acid target. Heat killed sample represents, the sample containing stop solution which was kept at 95 °C for 10 min, in order to fully inactivate any proteinase K activity. Ct values of 40 represents samples with residual pK activity, thereby inhibiting the reverse transcription (RT) reaction. Control Xeno only represents samples in which Xeno RNA was spiked into deionized water. Lysis solution represents the only PK solutions without inhibitor. No template control (NTC) reaction represents a reaction in which no Xeno template was added.

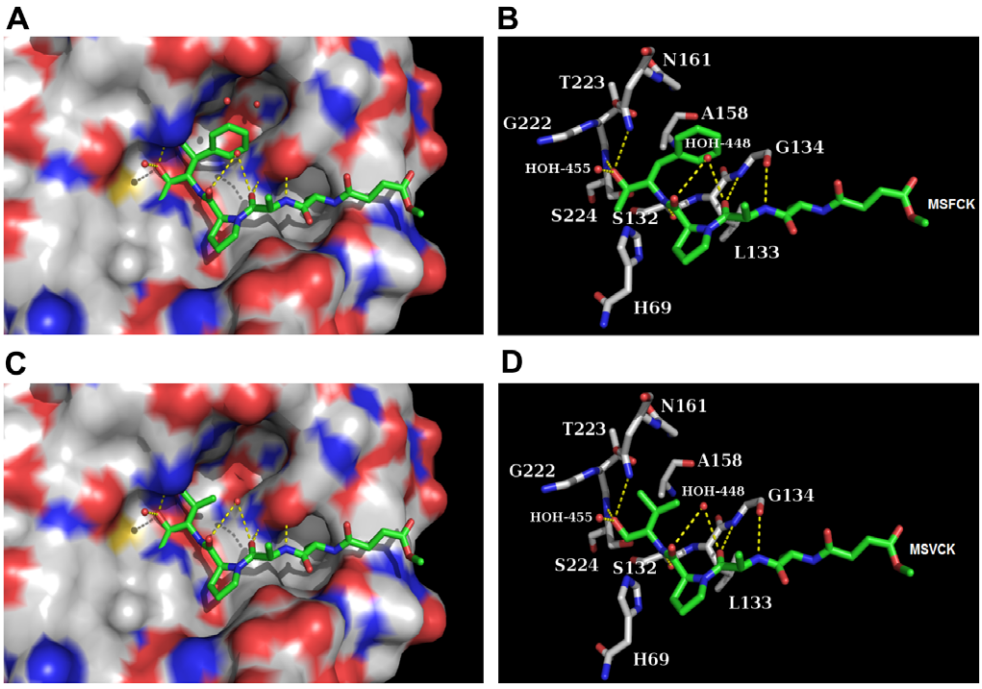


Figure 3. Screening for potential steric clashes of modified tetrapeptides MeOSuc-AAPV-CH_2Cl **1** and MeOSuc-AAPF-CH_2Cl **7** by molecular modeling. The coordinates of proteinase K in complex with MeOSuc-AAPA-CH_2Cl at 2.2 Å resolution were used as a starting point. (A and B) Solvent accessible surface of enzyme with methoxysuccinyl-Ala-Ala-Pro-Phe-chloromethyl ketone, (MSFCK) **7** modeled into the active site. (B) Identical to (A), except the molecular surface has been removed to highlight the protein–inhibitor interactions. (C) Solvent-accessible surface of enzyme with methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, (MSVCK) **1**. (D) Identical to (C), except the molecular surface has been removed to highlight the protein–inhibitor interactions.[14, 15]

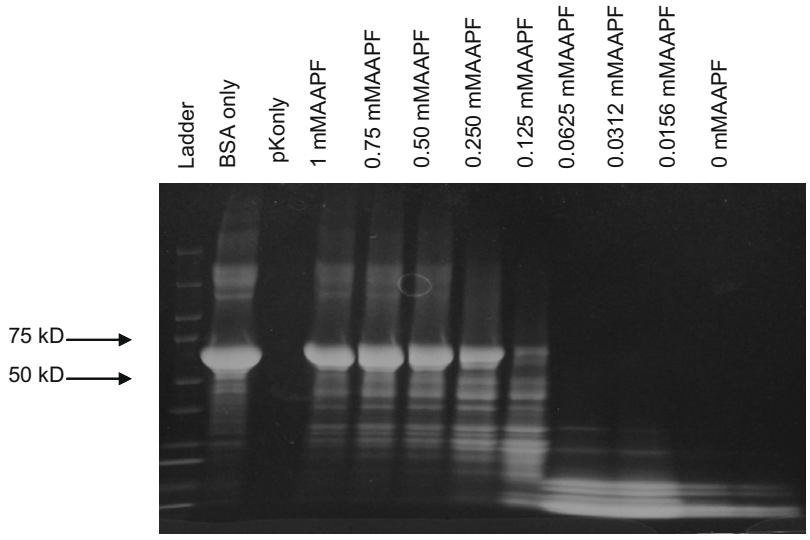


Figure 4. Independent digestion of proteinase K in presence of BSA and MeOSuc-Alal-Ala-Pro-Phe-CH_2Cl 7. Precast protein gel was run at 120 V for 1 h by using 1× Tris/glycine/SDS (Bio-Rad). Gel was stained with Coomassie blue stain for 30 min at room temp and destained with destaining solution (20% acetic acid, 10% methanol, and diluted to 1 l). Gel was exposed on Alpha Ease (FluoroChem SP).

the case of MeOSuc-AAPV-CH_2Cl **1**, the valine moiety has an aliphatic side chain that is slightly away from the binding pocket that makes it less connected to the active site than MeOSuc-AAPF-CH_2Cl **7**. It is interesting to compare the proteinase K inhibitory effect of MeOSuc-AAPF-CH_2Cl **7**, in which proline residue in the P2 position and MeOSuc-APAF-CH_2Cl **11**, in which proline residue in the P3 position. The data reveal that MeOSuc-AAPF-CH_2Cl **7** is a good inhibitor for the proteinase K, where as MeOSuc-APAF-CH_2Cl **11** loses the inhibitor effect. Although the exact reason for this reactivity difference is not entirely clear, it appears that the sequence of the tetra-amino acid fit (i.e., phenylalanine and proline residue are next to each other) is critical for the successful inhibitor activity.

The high proteinase K inhibitory activity of MeOSuc-AAPF-CH_2Cl **7** was further confirmed by an independent assay looking at the digestion of BSA by proteinase K solution in the presence of each of the putative inhibitors. In order to determine the concentration of MeOSuc-AAPF-CH_2Cl **7** and MeOSuc-AAPV-CH_2Cl **1** for effective inhibition of proteinase K, independent digestion assay with PK solution and Ultrapure BSA was carried our.[18] Figure

4 provides data from a protein gel in which the marker ladder ranges in size from 10 to 250 kDa, intact Ultrapure BSA substrate (Applied Biosystems) is at about 70 kDa, and proteinase K is at about 35 kDa. Figure 4 shows that MeOSuc-AAPF-CH_2Cl **7** was capable of inhibiting proteinase K (100 μg/ml) at concentrations as low as 0.125 mM, while MeOSuc-AAPV-CH_2Cl **1** was only capable of inhibiting proteinase K (100 μg/ml) at a concentrations as low as 0.25 mM. (Data not shown)

In conclusion, we have synthesized and evaluated the new potent proteinase K inhibitor MeOSuc-AAPF-CH_2Cl **7**, which inhibits proteinase K activity at 10-fold lower concentrations than the currently-known inhibitor, MeOSuc-AAPV-CH_2Cl **1**. Computer modeling studies indicated the presence of an additional small binding pocket in the vicinity of the proteinase K active site, which we postulate is filled by the aromatic phenylalanine moiety. The phenylalanine residue aromatic ring, which fits into this binding pocket and potentially offers additional van der Waals, hydrogen bonding, and/or hydrophobic interactions, enhances the inhibitor potency. We believe that the new inhibitor would be especially useful in molecular biology applications, especially sample preparation areas, where proteinase K used to digest away cellular components including nucleases can be inhibited in situ without its removal, and as a result, it will allow RT-qPCR to be subsequently performed in the same reaction mixture. In addition, due to the runaway protease activity occurring in some pathogenic situations, this molecule may have applications in the medical field in the future as well.

Acknowledgments

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9. *Data for MeOSuc-AAPF-CH_2Cl **7**:* ^{1}H NMR (D_2O, 400 MHz) δ 7.76 (m, 2H), 7.26–7.11 (m, 5H), 6.43 (d, *J* = 7.6 Hz, 1H), 4.78 (m, 1H), 4.64 (m, 2H), 4.49 (m, 1H), 4.10 (m, 2H), 3.72 (m, 1H), 3.65 (s, 3H), 3.56 (m, 1H), 3.11 (m, 1H), 2.95 (m, 1H), 2.59 (m, 2H), 2.47 (m, 2H), 2.14–1.93 (m, 4H), 1.35 to 1.19 (m, 6H); MS (*m*/*z*): 551 $[M+H]^+$.
10. *Data for MeOSuc-APAF-CH_2Cl **11**:* ^{1}H NMR (D_2O, 400 MHz) δ 7.27 to 7.07 (m, 7H), 6.52 (d, J = 6.8 Hz, 1H), 4.81 (m, 1H), 4.61 (m, 1H), 4.35 (m, 2H), 4.28 (d, *J* = 16.4 Hz, 1H), 4.04 (d, *J* = 16.4 Hz, 1H), 3.72 (m, 1H), 3.64 (s, 3H), 3.54 (m, 1H), 3.16 (m, 1H), 2.96 (m, 1H), 2.61 (m, 2H), 2.50 (m, 2H), 2.11–1.81 (m, 44), 1.32 (d, *J* = 6.8 Hz, 3H), 1.21 (d, J = 7.2 Hz, 3H); MS (*m*/*z*): 551 $[M+H]^+$.
11. *Preparation of stop solution with inhibitor:* Typically, stop solution was prepared in 100-μl volumes, containing the following components: Tris pH 8.0, 11 mM; EGTA pH 9.0, 88 mM; compound **1**, **7**, or **11**, at 11 mM; placental RNase inhibitor protein, 2.2 U/μl; and DTT, 0.11 mM. The solution pH was adjusted to 8.0, if required, by dilute HCl. A dilution solution was prepared identically, except that inhibitor analog was omitted. A series of solutions was made for each inhibitor were made by diluting the initial solutions with the dilution solution so that the final concentrations were 11, 8.25, 5.5, 2.75, and 1.10 mM (0, 25%, 50%, and 75% dilutions).
12. *Proteinase K treatment of reverse transcription reactions.* All reactions were carried out in triplicate. Fifty microliters of proteinase K solution (without DNase mixed in) from the TaqMan® gene expression Cells-to-CT™ kit (Applied Biosystems) were aliquoted into 18 tubes. To each of these (these are equivalent to lysis mixtures in the Cells-to-CT™ workflow), 5 μl of the various stop solutions was added, resulting in 1, 0.75, 0.5, 0.25, and 0.11 mM concentrations of the inhibitor analogs in the proteinase K solution. These were incubated 2 min at room temperature. For the control reaction, 50 μl of proteinase K solution was mixed with 5 μl of the dilution solution and heated at 95 °C for 10 min to thermally inactivate the proteinase K reaction. Next, the reverse transcription reagents were prepared, that is, 15 μl 2× RT buffer, and 1.5 μl of 20× RT enzyme mix, were aliquoted and mixedin the wells of a 96-well plate. To this solution, 13.5 μl of each proteinase K + stop mixture was added and the solution was mixed well. The resulting mixtures, containing 0.45, 0.3375, 0.224, 0.1125, and 0.045 mM of the appropriate analog was incubated at room temperature for 1 h for residual PK activity to degrade the RT enzyme. After 1 h incubation at room temperature, 1 μl of Xeno RNA (diluted to 25,000 copies/μl in 10 ng/μl poly A) was added to all wells except for the no template control wells. Then the reverse transcription (RT) reaction was incubated at 37 °C for 60 min, 95 °C for 5 min, and cooled to 4 °C, using the GeneAmp® 9700 PCR system (Applied Biosystems).
13. *Real-time PCR:* Real-time PCR was performed by using the Xeno AOD (Assay on Demand) TaqMan® Cells-to-CT™ control kit, on a 7500 Fast Real-Time PCR system (Applied Biosystems). In each reaction, the equivalent of 12.5 μl of TaqMan GEX master mix mixed with 1.25 μl of 20× Xeno AOD was dispensed in each well of a 96-well plate. 11.25 μl of each RT reaction was then added to each well. For three control reactions, 11.25 μl of water was added to the three wells. Then the 96-well plate was run by using the gene expression parameters as per manufacturer's instruction.
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18. *Independent digestion assay of compound **7** and **1**:* For the direct assay of PK inactivation, 50 μl of proteinase K (100 μg/ml) solution was mixed with 5 μl of stop solution with varying amounts of compound **7** and **1** separately and incubated for 10 min at room temperature. Ten microliters of Ultrapure BSA (50 mg/ml) was added to each sample and the samples were incubated for 10 min at room temperature. The reaction mixture was heated for 30 min at 95 °C to inactivate any remaining functional PK and the sample mixes analyzed using precast protein gels. For each 65 μl of total reaction mixture, 10 μl was mixed with 5 μl of gel-loading dye and further heated at 95 °C for 5 min. Then, the samples were kept on ice for 2 min and 15 μl of reaction mixture was loaded on the gel along with ladder. For the control reactions, 50 μl of PK solution without BSA, and 10 μl of BSA were mock-incubates and analyzed in a similar fashion.