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Design, synthesis and inhibitory effect of pentapeptidyl chloromethyl ketones on proteinase K

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ABSTRACT

The synthesis and proteolytic inhibitor function of new modified pentapeptide MeOSuc-AAAPF-CH_2Cl **6** is described. The efficacy of **6** in inhibiting the proteolytic activity of proteinase K at a concentration of 0.10 mM allows a signal to be obtained for an exogenous target ('Xeno RNA') at 29 PCR cycles (i.e., Ct = 29), whereas the control MeOSuc-AAAPV-CH_2Cl **1** requires a 7.5-fold higher concentration (0.75 mM) to produce the same Ct.

1. Introduction

Proteases are otherwise called proteolytic enzymes which selectively catalyze the hydrolysis of peptide bonds that are involved in a number of disease states.[1] In particular, the target serine proteases play a crucial role in a broad spectrum of physiological process such as food digestion, protein turn over, blood coagulation, wound healing, fertilization, cell differentiation and growth, cell signaling, immune response, and apoptosis.[1–4] It is believed that uncontrolled, unregulated or undesired proteolysis leads to many disease states such as emphysema, arthritis, stroke, viral infections, cancer, Alzheimer's disease, and inflammation. Selective regulation of these and other proteases in the disease state is an important clinical problem.[5] It is noteworthy that several protease inhibitors have entered into advanced clinical trials.[6,7] Consequently, medicinal chemistry efforts aiming at the discovery of chemical inhibitors of the serine protease are currently pursued in the biotech industries as well as academia.

The use of peptidyl chloromethyl ketones has been demonstrated as the first active site-directed irreversible inhibitors for any enzyme.[8] In addition, it has been reported as the first affinity label for serine proteases.[9] While several classes of inhibitors such as halenol lactones,[10] substituted 6-chloro-2-pyrones,[11] *N*-nitro lactams,[12] halomethylcoumarins,[5] and 2-bromomethyl-3,1-benzoxazin-4-one[13] have been developed, the most commonly used active-site serine protease is the use of peptidyl chloromethylketones. There are two special structural features on peptidyl chloromethyl ketones that play an important role in the specific interaction of inhibitor–enzyme complex based on the X-ray crystallographic data.[14–16] First, the presence of chloromethyl ketone group is necessary that covalently linked with the active site functional groups $His^{69}(N_\varepsilon)$ and $Ser^{224}(O_\gamma)$. Second, the peptide chain of the inhibitor is inserted into the recognition site such that an antiparallel three stranded β-pleated sheet is formed. It also helps to increase the resemblance of the inhibitor to enzymes. The effect of the chain length[17] and the nature of the amino acid at P1 position[18] on the peptidyl chloromethyl ketones appear to have a significant impact on the proteinase K activity. In continuation of our search efforts[18,19] to find better inhibitors to eliminate the need for proteinase K removal prior to further in vitro enzymatic reactions prompted us to explore the possibility of using pentapeptidyl chloromethyl ketones for inhibiting serine proteases. The modified pentapeptides 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 penta-amino acid chain. From the X-ray crystallographic studies, it appears that the presence of the methoxysuccinyl group aids binding to the recognition site of proteinase K by two hydrogen bonds, one directly between the methoxy oxygen of the succinyl group and the hydrogen of the Tyr^{104} (N), and the other mediated by a water molecule, succinylpeptide (C+O)...Wat^{453}...(HN)Gly^{136}.[14] In this article, we report the synthesis and biological validation of new modified pentapeptide MeOSuc-AAAPF-CH_2Cl (AAAPF) **6** by using MeOSuc-AAAPV-CH_2Cl (AAAPV) **1**[19] as control against proteinase K validation. The chemical structure of control inhibitor MeOSuc-AAAPV-CH_2Cl **1** was represented in Figure 1.

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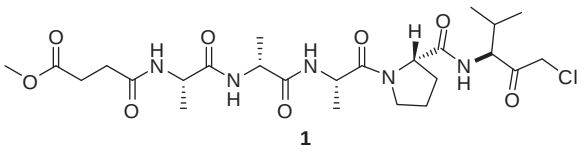
2. Results and discussion

2.1. Synthesis

The designed pentapedptidyl chloromethyl ketone MeOSuc-Ala-Ala-Ala-Pro-Phe-CH_2Cl **6** was synthesized through a three step synthetic route as shown in Scheme 1. The peptide coupling reaction of **2** with 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-AAAPF-CH_2Cl **4** in 89% yield. The cleavage of Boc group was accomplished using 6 M HCl/EtOAc system to afford the corresponding H-AAAPF-CH_2Cl·HCl **5** in 91% yield. Finally, treatment of **5** with methyl succinimido succinate in the presence of THF/$NaHCO_3$ system furnished corresponding **6** in 79% yield. The structure of **6** was confirmed by NMR and mass data.

2.2. Biological validation

The proteinase K activity of new analog **6** was examined using a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay. For this purpose, we have used the TaqMan® gene expression Cells-to-CT™ kit and TaqMan® Cells-to-CT™ control kit (Life Technologies Corporation). The stop solutions with and without inhibitors **1** and **6** were prepared and reverse transcription and real time PCR were carried out as described in Section 4. The outcome of the analysis of RT and qPCR with new proteinase K inhibitor **6** was compared with the standard inhibitor **1** as a control and their average Ct values plotted against the various concentrations of the pentapeptides (Fig. 2).



1

Figure 1. Chemical structure of MeOSuc-Ala-Ala-Ala-Pro-Val-CH_2Cl **1**.

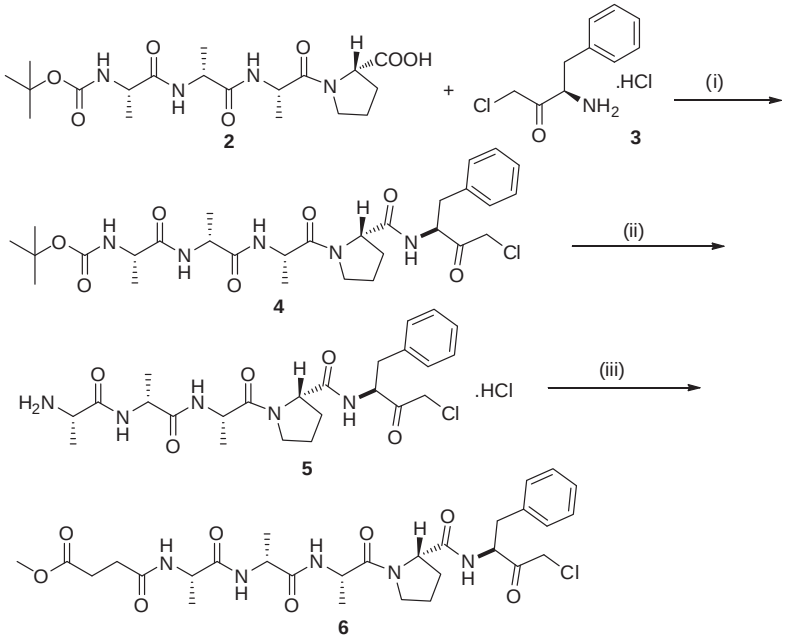
As shown in Figure 2, compound **6** showed proteinase K inhibition at a series of concentrations from 0.1 to 1 mM, resulting in a Ct of about 29, whereas the control showed inhibition at concentrations higher than 75 mM to produce the same Ct. These data clearly indicate that the new inhibitor **6** is 7.5 times more active than the control **1**.

Encouraged by the higher proteinase K inhibitory activity of modified pentapeptide **6**, we then validated by in vitro detection of β-actin RNA in HeLa Cells (Fig. 3). In this protocol, HeLa cells (~10,000 cells) were lysed in 50 μL lysis solution for 5 min at room temperature. Stop solution (5 μL) was then added; the lysate-stop solution was mixed, and incubated for 2 min at room temperature. A volume of 10 μL of stopped lysate was added to a 50 μL RT reaction followed by addition of 4 μL of RT reaction into a 20 μL PCR reaction using the β-actin primers/probe of the TaqMan® Gene Expression Cells-to-CT™ Kit (Life Technologies Corporation). Notably, compound **6** inhibits proteinase K at a concentration of 0.10 mM, whereas control **1** inhibited at a concentration of 0.5 mM.

The high proteinase K inhibitory activity of **6** 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. A direct assay for inhibition of proteinase K activity by **6** was carried out using bovine serum albumin (BSA) as substrate and analyzing products using precast protein gels.

The outcome of the direct assay (Fig. 4) shows that **6** was capable of inhibiting proteinase K (100 μg/mL) at concentrations as low as 0.25 mM, while **1** was not capable of inhibiting proteinase K (100 μg/mL) at a concentrations as high as 0.75 mM. This assay demonstrate that the results of the indirect linked assay of MeO-Suc-AAAPF-CH_2Cl **6** and MeOSuc-AAAPV-CH_2Cl **1** are due to inhibition of the proteinase K by the test inhibitors and are not due to an inhibition of the RT-PCR reaction.

From the present study, it seems that the modification at the P1 residue on pentapeptidyl chloromethyl ketones has had a significant effect on the proteinase K activity. It is noteworthy that the presence of phenylalanine at P1 position **6** inhibits at 7.5-fold lower concentrations than the corresponding isomeric compound with



Scheme 1. Reagents and conditions: (i) isobutyl chloroformate; *N*-methymorpholine; THF, 4 °C, 15 h; (ii) 6 N HCl/EtOAc, rt, 3 h; (iii) methyl succinimido succinate, THF/$NaHCO_3$, rt, 3 h.

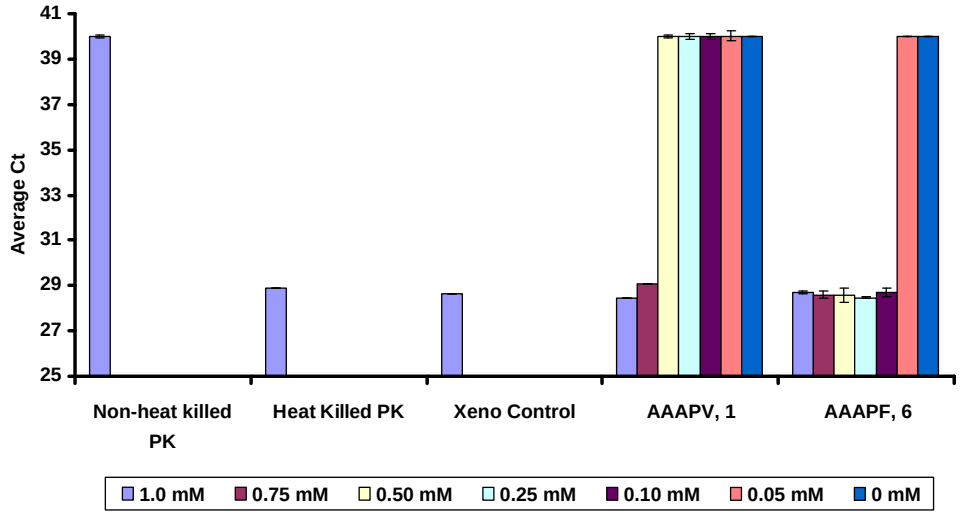


Figure 2. Histograms of average Ct (cycle threshold) values were plotted against the varying concentration of control MeOSuc-AAAPV-CH_2Cl **1**, and MeOSuc-AAAPF-CH_2Cl **6**. The term "Ct" represents the PCR cycle number when the signal is first recorded as statistically significant.

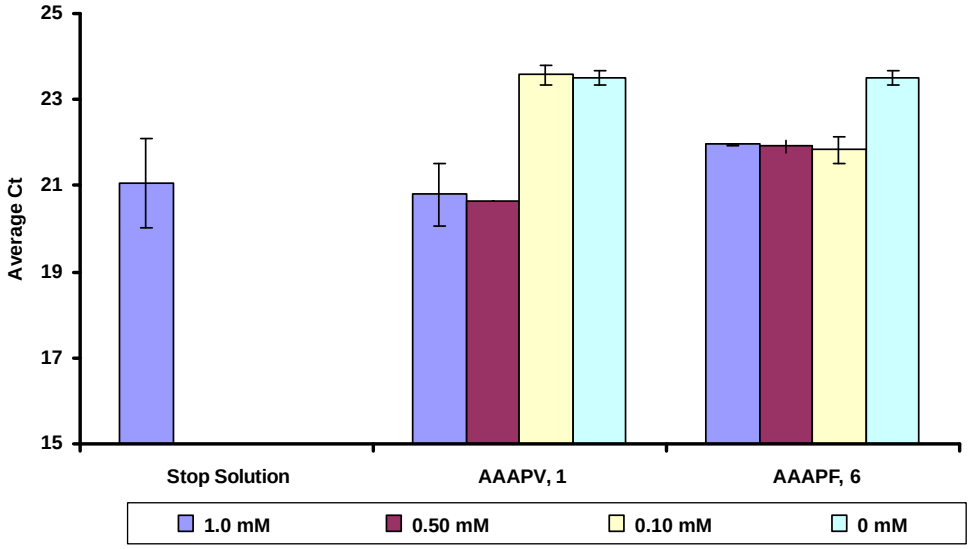


Figure 3. In vitro validation data showing the detection of β-actin in HeLa cell cultures.

a valine at P1 position **1**. This result is in similar agreement with the results obtained for the modification of P1 residue on tetrapeptidyl chloromethyl ketones in which the phenylalanine analog (MeOSuc-AAPF-CH_2Cl) is 10 times more active than the corresponding valine analog (MeOSuc-AAPV-CH_2Cl).[18] The remarkable proteinase K inhibition of **6** over **1** may be understood in terms of the nature of the amino acid at P1 position. While, the exact reason for the reactivity difference is not clear it seems that aromatic amino acid, phenylalanine at P1 position makes more stable complex between phenylalanine residue and proteinase K than the aliphatic amino acid, valine at P1 position.

3. Conclusion

In summary, we have synthesized and evaluated the inhibitory efficiency of pentapeptidyl chloromethyl ketone such as AAAPF **6**. The presence of phenyl alanine at P1 position appears to be critical for the higher proteinase K activity compared to the presence of valine at P1 position. Considering the superior inhibitory efficiency of **6** over the control **1**, one can expect their applications in molecular biology, such as in qRT-PCR, where inhibition of proteinase K is required after their role in digesting cellular components. In addition, due to the runaway protease activity occurring in some pathogenic situations, this inhibitor **6** may find applications in the medical field in the future as well.

4. Experimental

4.1. Chemistry

^1H NMR spectra were recorded in $CDCl_3$ on a Bruker 400. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). ESI mass was recorded on a Applied Biosystems/Sciex MDX API 150 model. HPLC was run on a Waters 2996 (Waters Corporation) using anion-exchange column.

4.1.1. Preparation of Boc-AAAPF-CMK 4

To a stirred solution of Boc-Ala-Ala-Ala-Pro-OH **2** (0.5 g, 1.17 mmol) in 10 mL of THF under argon atmosphere at 4 °C,

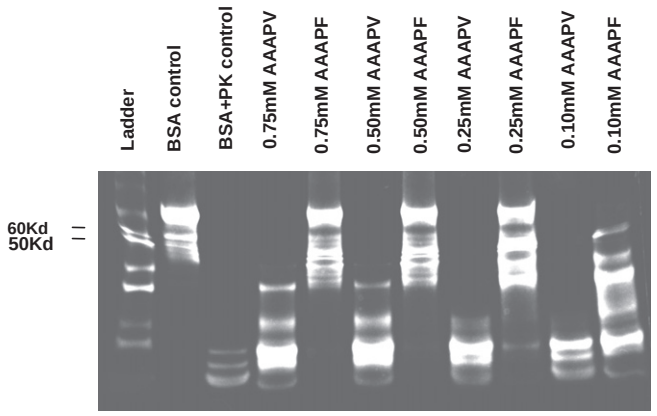


Figure 4. Independent digestion of proteinase K in presence of BSA and the inhibitors **1** and MeOSuc-AAAPF-CH_2Cl **6**.

isobutyl chloroformate (0.23 mL, 1.76 mmol) and *N*-methylmorpholine (0.51 mL, 4.65 mmol) were added and the reaction mixture was allowed to stir for 15 min. After 15 min. a solution of Phe-CMK·HCl **3** (0.38 g, 1.63 mmol) in 10 mL of THF was added by syringe over 15 min. The resulting reaction mixture was allowed to stir for 15 h and diluted with dichloromethane (50 mL). The resulting solution was washed successfully with 10 mL of 1 N HCl, 10 mL of 5% $NaHCO_3$, and 10 mL of 2 M NaCl solution. The collected organic layer was dried over $MgSO_4$, filtered and concentrated under rotary evaporator. To the resulting residue, 50 mL hexane was added and stirred for 10 min. After stirring for 10 min filter the resulting suspension and dried under vacuum for 10 min to give Boc-AAAPF-CMK **4** (0.63 g, 89%) as a pale yellow colored solid.

4.1.2. Preparation of H-AAAPF-CMK·HCl 5

To a stirred solution of Boc-AAAPF-CMK **4** (0.62 g, 1.02 mmol) in 10 mL of ethyl acetate, 10 mL of 6 N HCl was added. The resulting reaction mixture was allowed to stir at rt for 3 h. After 3 h, the resulting mixture was washed with 50 mL dichloromethane. The collected aqueous solution was evaporated under rotor evaporator to give H-AAAPF-CMK·HCl **5** (0.51 g, 91%) as oil. This crude product was used for next step without further purification.

4.1.3. Preparation of MeoSuc-AAAPF-CMK 6

To a stirred solution of H-AAAPF-CMK·HCl **5** (0.51 g, 0.94 mmol) in 10 mL of THF, a suspension of sodium bicarabonate (1.0 g in 1 mL water) was added and allowed to stir at for 10 min. After 10 min, methyl succinimido succinate (0.19 g, 0.83 mmol) was added and the resulting reaction mixture was allowed to stir at rt for 3 h. After 3 h, the reaction mixture was diluted with dichloromethane (50 mL). The resulting organic layer was washed with water (15 mL) and brine (15 mL). The organic layer was evaporated under rotary evaporator to give the pure MeoSuc-AAAPF-CMK **6** (0.41 g, 79%) as a white colored solid. Data for MeoSuc-AAAPF-CMK: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.57 (d, J = 8.0 Hz, 1H), 7.47 (d, J = 5.6 Hz, 1H), 7.29–7.13 (m, 5H), 7.05 (d, J = 8.4 Hz, 1H), 6.38 (d, J = 6.0 Hz, 1H), 4.71 (m, 1H), 4.61 (m, 1H), 4.46 (m, 2H), 4.36 (d, J = 16.8 Hz, 1H), 4.32 (m, 1H), 4.15 (d, J = 16.8 Hz, 1H), 3.65 (m, 1H), 3.63 (s, 3H), 3.56 (m, 1H), 3.21 (m, 1H), 2.87 (m, 2H), 2.61 (m, 1H), 2.46 (m, 2H), 2.01–1.81 (m, 4H), 1.41 (d, J = 7.2 Hz, 3H), 1.36 (d, J = 7.6 Hz, 3H), 1.31 (d, J = 6.8 Hz, 3H); MS (m/z): 622 $[M+H]^+$.

4.2. Biology validation

4.2.1. 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; MeoSuc-AAAPV-CH_2Cl **1** and **6** separately in each tube at final concentration of 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 1 mM (100%), 0.75 mM (75%), 0.5 mM (50%), 0.25 mM (25%), 0.10 mM (10%), 0.05 mM (5%) and control 0 mM (0%).

4.2.2. Reverse transcription reactions

All reactions were carried out in triplicate. 50 μL 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 of MeoSuc-AAAPV-CH_2Cl **1** and **6** were added separately in each tube, resulting in 1, 0.75, 0.5, 0.25, 0.10, 0.05 mM and control 0 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 mixed in 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 various concentration of the appropriate analog were 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).

4.2.3. 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.

4.2.4. Independent digestion assay of 1 and 6

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 **1** and **6** separately and incubated for 10 min at room temperature. 10 μL of Ultrapure BSA (5 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.

4.2.5. Protein gel

The precast protein gel was run at 120 V for 1 h using 1× Tris/glycine/SDS buffer (BIO-RAD, Hercules, CA). The gel was stained

with Coomassie blue stain for 1 h at room temp and destained with destaining solution (20% acetic acid, 10% methanol in 1 L). The gel was analyzed using AlphaEase™ FC software (Alpha Innotech, San Leandro, CA). The protein ladder ranges in size from 10 to 260 kDa (Novex® Sharp Pre-Stained Protein Standard (Invitrogen, Carlsbad CA).

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References and notes

1. Powers, J. C.; Asgian, J. L.; Ekici, Z. D.; James, E. *Chem. Rev.* **2002**, *102*, 4639.
2. Tyndall, J. D.; Nall, T.; Fairlie, D. P. *Chem. Rev.* **2005**, *105*, 973.
3. Turk, B. *Rev. Drug Disc.* **2006**, *5*, 785.
4. Thurmond, R. L.; Edwards, J. P. *Drug Discov. Ser.* **2006**, *5*, 113 (Handbook of Assay Development in Drug Discovery).
5. Harper, J. W.; Hemmi, K.; Power, J. C. *Biochemistry* **1985**, *24*, 1831.
6. Clynen, E.; Schoofs, L.; Salzet, M. *Med. Chem. Rev. Online* **2005**, *2*, 197.
7. Maryanoff, B. E.; Costanzo, M. J. *Bioorg. Med. Chem.* **2008**, *16*, 1562.
8. Shaw, E.; Mares-Gula, M.; Cohen, W. *Biochemistry* **1965**, *4*, 2219.
9. Schoellmann, G.; Shaw, E. *Biochemistry* **1963**, *2*, 252.
10. Chakravarty, P. K.; Krafft, G. A.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1982**, *257*, 610.
11. Westkaemper, R. B.; Abeles, R. H. *Biochemistry* **1983**, *22*, 3256.
12. White, E. H.; Jelinski, L.; Politzer, I. R.; Branchini, B. R.; Roswell, D. F. *J. Am. Chem. Soc.* **1981**, *103*, 4231.
13. Alazard, R.; Bechet, J. J.; Dupaix, A.; Yon, J. *Biochim. Biophys. Acta* **1973**, *309*, 379.
14. Wolf, W. M.; Bajorath, J.; Muller, A.; Raghunathan, S.; Singh, T. P.; Hinrichs, W.; Saenger, W. *J. Biol. Chem.* **1991**, *266*, 17695.
15. Wilcox, P. E.; Segal, D. M.; Powers, J. C.; Cohen, G. H.; Davies, D. R. *Biochemistry* **1971**, *10*, 3728.
16. Robertus, J. D.; Kraut, J.; Alden, R. A.; Birktoft, J.; Subtilsin, J. *Biochemistry* **1972**, *11*, 4293.
17. Powers, J. C.; Tuhy, P. M. *Biochemistry* **1973**, *12*, 4767.
18. Kore, A. R.; Shanmugasundaram, M.; Hoang, Q.; Kuo, M.; Chapman, L. M.; Chen, H. H. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1296.
19. Kore, A. R.; Shanmugasundaram, M.; Charles, I.; Hoang, Q. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2750.