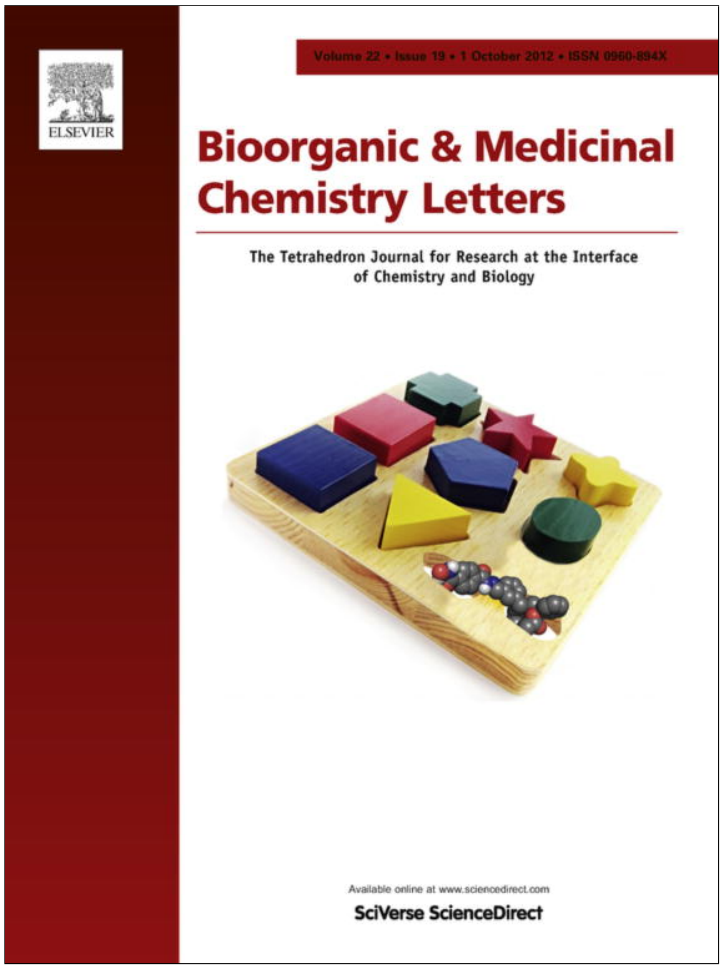


Bioorganic & Medicinal Chemistry Letters 22 (2012) 6072–6075

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Isomannide derivatives as new class of inhibitors for human kallikrein 7

Renato F. Freitas [a,†], Thiago S. P. Teixeira [a,†], Thalita G. Barros [b], Jorge A. N. Santos [c], Marcia Y. Kondo [d], Maria A. Juliano [d], Luiz Juliano [d], Michael Blaber [e], Octávio A. C. Antunes [f], Odonírio Abrahão Jr. [a], Sergio Pinheiro [g], Estela M. F. Muri [b], Luciano Puzer [a,h,*]

[a] Instituto de Ciências Biológicas e Naturais, Universidade Federal do Triângulo Mineiro, Uberaba, MG, Brazil
[b] Faculdade de Farmácia, Universidade Federal Fluminense, Niterói, RJ, Brazil
[c] Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais, Campus Inconfidentes, Brazil
[d] Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, SP, Brazil
[e] Department of Biomedical Sciences, Florida State University, Tallahassee, FL, USA
[f] Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[g] Instituto de Química, Universidade Federal Fluminense, Niterói, RJ, Brazil
[h] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Rua Santa Adélia 166, Bairro Bangu, 09210-170, Santo André, SP, Brazil

ARTICLE INFO

Article history:
Received 26 May 2012
Revised 3 August 2012
Accepted 13 August 2012
Available online 21 August 2012

Keywords:
Serine protease
Kallikrein
Pseudo-peptide
Inhibitor
Molecular dynamics

ABSTRACT

Human kallikrein 7 (KLK7) is a potential target for the treatment of skin inflammation and cancer. Despite its potential, few KLK7-specific small-molecule inhibitors have been reported in the literature. As an extension of our program to design serine protease inhibitors, here we describe the in vitro assays and the investigation of the binding mechanism by molecular dynamics simulation of a novel class of pseudo-peptide inhibitors derived from isomannide. Of the inhibitors tested, two inhibited KLK7 with K_i values in the low micromolar range (**9g** = 1.8 μM; **9j** = 3.0 μM). Eadie–Hofstee and Dixon plots were used to evaluate the competitive mechanism of inhibition for the molecules. Calculated binding free energies using molecular MM/PB(GB)SA approach are in good agreement with experimental results, suggesting that the inhibitors share the same binding mode, which is stabilized by hydrophobic interactions and by a conserved network of hydrogen bonds. The promising results obtained in this study make these compounds valid leads for further optimization studies aiming to improve the potency of this new class of kallikrein inhibitors.

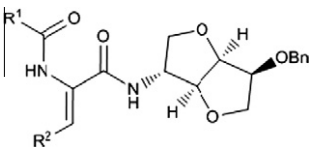
Human tissue kallikreins consist of 15 kallikrein-like serine peptidases (KLK1-KLK15).[1–3] Kallikrein 7 (KLK7) is one of the family members that exhibit chymotrypsin-like activity. It is most abundantly expressed in the skin and has been reported to play an important role in skin physiology.[4,5] The desquamation behavior of human stratum corneum (SC) has been associated with the progressive degradation of corneodesmosomes toward the outer skin surface. This process is facilitated by the action of specific enzymes in the SC, including kallikrein 7.[6] Therefore, KLK7 is considered to be a potential target for the treatment of diseases involved with epithelial dysfunction, such as inflammatory and/or hyperproliferative and pruritic skin diseases, including atopic dermatitis, psoriasis, and Netherton's syndrome.[7–11] Besides the essential role of KLK7 in the skin desquamation process, its involvement in tumor metastasis, especially in ovarian carcinomas and pancreatic cancer, has also been investigated. While this enzyme is moderately expressed in the normal ovary, it is overexpressed in ovarian carcinoma tissues at the mRNA and/or protein levels. The results of an in vivo model strongly suggest that the overexpression of KLK4, KLK5, KLK6, and KLK7 contributes to ovarian cancer progression.[12] Also, differences in the expression of KLK7 could potentially be used as biomarkers for the characterization of different stages of cervical neoplasia.[13]

Recently, our efforts to find new KLKs inhibitors from natural products resulted in the identification of two isocoumarins (Vioxanthin and 8,8′-paepalantine) that inhibited KLK5 and KLK7 in the low micromolar range.[14] Thus, as a part of our program of developing new serine protease inhibitors, here we present the results of the in vitro assays against KLK7 and molecular dynamics simulation studies with a series of isomannide analogue compounds (Table 1). The use of the isomannide rigid scaffold was envisaged due to its structural analogy with cyclic rigid dipeptides.[15–19] The rigidity of this scaffold allows the compound to be fixed in its bioactive conformation. The synthesis of the isomannide analogue compounds was previously described by Muri et al.[15] The mature human tissue kallikreins were expressed as recombinant proteins from an insect cell/baculovirus expression system, as described previously.[14] The Fluorescence Resonance

* Corresponding author. Tel.: +55 11 4996 8388.
E-mail address: luciano.puzer@ufabc.edu.br (L. Puzer).
† These authors contributed equally to this work.

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.08.047

Table 1
Structure of isomannide derivatives with various substituents at R1 and R2, along with their IC_{50} and K_i values (expressed in μM). NS, not soluble in aqueous buffer



Compound	R^1	R^2	KLK7		KLK5	
			IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
9a	Ph	Ph	NS			
9b	Ph	4-FC_6H_4	NS			
9c	Ph	4-CLC_6H_4	NS			
9d	Ph	4-BrC_6H_4	NS			
9e	Ph	4-F_3CC_6H4	NS			
9f	Ph	4-$MeOC_6H_4$	NS			
9g	Ph	2-Thiophene	13.3	1.8	99.2	70.7
9h	Ph	3,4-$(OCH_20)C_6H_3$	205.2	80.3	>1000	357.1
9i	Ph	3-Pyridyl	195.8	75.1	323.5	200
9j	Ph	2-Furan	16.3	3.0	99.1	70.4
9k	Me	2-Thiophene	160.5	77.4	101.3	71.9
91	Ph	1-Benzothiophen-2-yl	NS			
9m	Ph	2-Naphthyl	NS			
9n	Ph	lH-Indol-3-yl	NS			
9o	Ph	Benzofuran-2-yl	NS			

Energy Transfer (FRET) peptides Abz-KLYSSKQ-EDDnp (Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine) and Abz-KLRSSKQ-EDDnp were synthesized by solid phase synthesis methods, as described previously.[20] All enzymatic reactions were performed in 50 mM Tris–HCl, pH 7.5.

Of the 15 compounds assayed, five (**9g**, **9h**, **9i**, **9j** and **9k**) were soluble in aqueous buffer and able to be assayed against KLK7. The other compounds were insoluble even in 10% DMSO. Of the five compounds assayed, two presented IC_{50} values below 50 μM. Compound **9g** was the most potent, with an IC_{50} of 13.3 μM, followed by compound **9j** (IC_{50} = 16.3 μM). To better understand the interaction between KLK7 and compounds **9g** and **9j**, we performed a detailed kinetic study to determine the mechanism of inhibition using the FRET substrate Abz-KLYSSKQ-EDDnp. The Eadie–Hofstee plot showed a simple mutually exclusive binding site between inhibitor and substrate (Fig. 1), establishing that the molecules behave as reversible competitive inhibitors for KLK7. As the IC_{50} values of competitive inhibitors can vary with substrate concentration, we also determined the values of the inhibition constants (K_i) for both compounds **9g** and **9j** using the Dixon plots (Fig. 1, inset), in which the reciprocal of the initial velocity ($1/V_0$) was plotted versus three different inhibitor concentrations (1.0, 2.0 and 4.0 μM), for four different concentrations of substrates (1.0, 2.0, 4.0 and 7.0 μM). We observed that the K_i values followed the same trend as the IC_{50} values, with compound **9g** being the most potent inhibitor, with a K_i of 1.8 μM, followed by compound **9j** (K_i = 3.0 μM). Compared with the IC_{50} values, the K_i values were five- to seven-fold lower for the two most potent compounds and approximately two fold lower for the other compounds. As the human tissue kallikrein 5 (KLK5) also appears to be over-expressed in some skin desquamation pathologies together with KLK7, we decide to evaluate our compounds also against KLK5. Inspecting the IC_{50} and K_i values for the inhibition of KLK5 reported in Table 1 we can see that these compounds are weak inhibitors of this enzyme. The best two compounds are also the **9g** and **9j** with a K_i values around 70 μM.

In order to rationalize differences in the affinity of the inhibitors for KLK7 and to explain why they are poor KLK5 inhibitors we performed molecular docking combined with molecular dynamics simulations to predict the binding mode of all compounds in the binding pocket of these two enzymes. The details of the site definitions, structure preparation and docking protocol were described in the Supplementary data. Binding modes of the newly found KLK7 inhibitors were addressed with Glide XP.[21] The docking results exhibited no correlation with the experimental affinities, ranking compound **9k** first, with a score of −5.87 kcal/mol, followed by the compounds **9g** (−5.84 kcal/mol) and **9j** (−5.68 kcal/mol). For KLK5, the docking scores also shows no agreement with the experimental results, ranking the compound **9g** first, with a slightly better score (−7.39 kcal/mol) than **9k** (−7.09 kcal/mol), while **9j** docked with the lowest score (−6.91 kcal/mol). These analyses show that docking scores could not rank order compounds and even it assigned incorrectly a higher affinity of the compounds for KLK5 contradicting the experimental results. Since the Glide scoring function was unable to differentiate individual active molecules from inactive ones we decide to apply molecular dynamics simulation combined with MM/PB(GB)SA (Molecular Mechanics/Poisson-Boltzmann Surface Area/Generalized Born Surface Area) as a more accurate method to investigate the protein-ligand interactions.[22–24] The binding mode of the complexes identified with Glide was used as starting structures for 5 ns of MD simulations (for details see Supplementary data). Inspection of the MD trajectories revealed that the backbone atoms of the KLK7 complexed with the three inhibitors showed almost identical and stable RMSD of ~1.0 Å compared to the initial structure, indicating that the protein had no obvious conformation changes during the MD simulations (Fig. 2). The inhibitor **9g** appeared to reach a stable state after 0.8 ns, indicating that this inhibitor underwent some conformational change or positional shift, but remained nearly constant for the rest of the trajectory, around 2.0 and 2.5 Å. In contrast, the RMSD of the inhibitors **9j** and **9k** remained approximately constant around the range of 2.5–3.0 Å (Fig. 2). For the complexes between the inhibitors and KLK5, the protein showed to be very stable during the 5 ns of simulation, with the RMSD for the backbone atoms in the range of 0.9–1.2 Å compared to the docked starting structure (Supplementary data). In the case of the inhibitors, it was evident that **9g** and **9j** have a more stable binding mode (RMSD~2 Å) in the KLK5 pocket than the **9k**

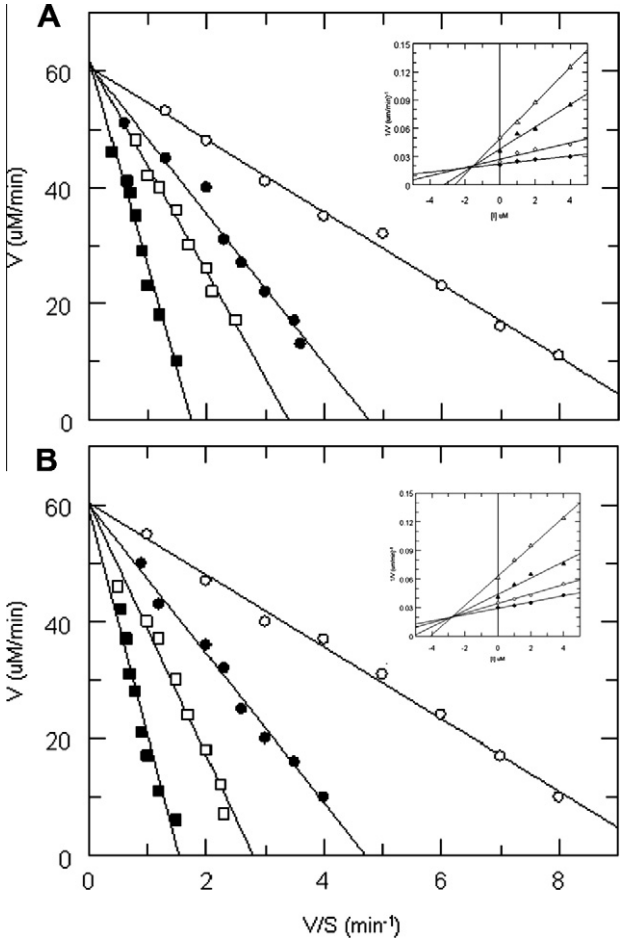


Figure 1. Representative Eadie–Hofstee (V vs V/[S]) plots for the hydrolysis of FRET substrates by KLK7 in the presence of inhibitor **9g** (A) and **9j** (B). Inset, the Dixon plots for both inhibitors against KLK7. Each data point corresponds to mean values ± S.E. of triplicate measures of a representative experiment. The solid lines represent the linear regression fits obtained by software GraFit 5.0.

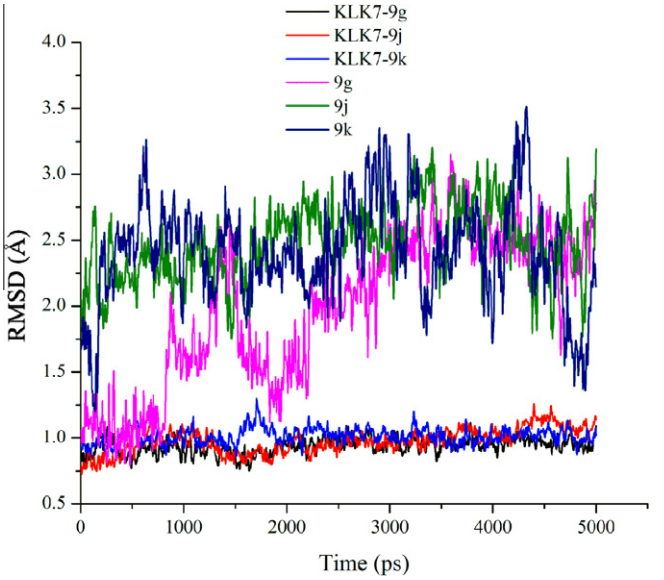


Figure 2. RMSD of the backbone atoms of KLK7 enzyme and heavy atoms of the inhibitors.

Table 2
Binding free energy components (kcal/mol) calculated with MM/GBSA and MM/PBSA

	KLK7			KLK5		
	9g	**9j**	**9k**	**9g**	**9j**	**9k**
			MM/GBSA			
ΔE_{vdw}[a]	−48.0	−43.2	−39.8	−50.8	−43.1	−38.0
ΔE_{ele}[b]	−29.0	−27.5	−19.4	−43.6	−21.6	−18.4
ΔC_{gb}[c]	42.1	40.3	36.8	57.5	39.3	37.3
ΔG_{SA}[d]	−5.5	−4.9	−4.2	−6.0	−5.0	−3.9
ΔG_{GBSA}[e]	−40.3	−35.4	−26.6	−42.9	−30.4	−23.0
			MM/PBSA			
$\Delta E_{vd}W$	−48.0	−43.2	−39.8	−50.8	−43.1	−38.0
ΔE_{ele}	−29.0	−27.5	−19.4	−43.6	−21.6	−18.4
ΔG_{GB}	50.8	50.0	45.0	73.8	49.5	44.6
ΔG_{SA}	−3.9	−3.5	−3.1	−4.2	−3.5	−2.6
ΔG_{PBSA}[e]	−30.1	−24.2	−17.3	−24.8	−18.8	−14.4
K_i	1.8	3.0	77.4	70.7	70.4	71.9
ΔG_{exp}[*]	−7.8	−7.5	−5.6	−5.7	−5.7	−5.6

[a] van der Waals interaction energy.
[b] Electrostatic interaction energy.
[c] Polar solvation energy calculated according the GB or PB approaches.
[d] Nonpolar solvation energy.
[e] Free energy of binding calculated according the GB or PB approaches.
[*] Experimental free energies of binding (kcal/mol) according to $\Delta G_{exp} = -RT\ln K_i$, where R and T are the gas constant, and temperature in Kelvin, respectively.

inhibitor, with this inhibitor reaching the equilibrium after 1.0 ns and fluctuating at an average of 3.0 Å (Supplementary data).

According to the above analysis, the MM/PB(GB)SA free energy calculation was based on 800 snapshots extracted from the MD trajectories between 1 and 5 ns at a time interval of 5 ps. The calculated binding free energy and its individual components are listed in Table 2. A significant correlation between the calculated binding free energies ($\Delta G_{PB(GB)SA}$) and the experimental affinity was observed. The ΔG_{GBSA} calculated according the MM-GB/SA method for compounds **9g**, **9j**, and **9k** complexed with KLK7 are −40.3, −35.4 and −26.6 kcal/mol, respectively. Using the MM-PBSA approach the values of ΔG_{PBSA} are −30.1, −24.2, and −17.3 kcal/mol, respectively. A closer analysis of the energetic terms showed that the main favorable contributions to the binding of inhibitors **9g** and **9j** came from van der Waals (ΔE_{vdW}) and electrostatic (ΔE_{ele}) terms. In contrast, the polar desolvation energy term ($\Delta E_{PB(GB)}$) is less favorable for these compounds than for **9k**, while the non-polar component (ΔE_{SA}) of solvation free energy was almost identical for all inhibitors. Independent of the method used, the decreasing order in the affinity (**9g** > **9j** > **9k**) for the KLK5-inhibitor complexes was the same as observed for KLK7. But according to the experimental results all inhibitors have nearly the same affinity for KLK5. The lack of variation in the compound's activity could be attributed to the low solubility of these compounds in the assay conditions, and not due their structures, which makes challenging to experimentally determine their true affinity against the enzyme. Additionally, the use of MM/PB(GB)SA proved to be a reliable approach to accurately predict the relative free energy of binding of the inhibitors for KLK7 and KLK5 enzymes. In contrast to the docking results, the inhibitors **9g** and **9j** were correctly identified by the MM/PB(GB)SA predictions as more potent inhibitors of KLK7 instead of KLK5 in line with our kinetics results (Table 2). The only exception was that the calculated ΔG_{GBSA} for the compound **9g** was lower in KLK7 than in KLK5 (−40.3 vs −42.9 kcal/mol).

In summary, this work describes the evaluation of five compounds against KLK7 and, in comparison, their activities against KLK5. Two of these molecules (**9g** and **9j**) inhibited kallikrein 7 activity in a dose-dependent manner, with IC_{50} values ranging from 13 to 200 μM. Subsequent determination of the mechanism of inhibition by Eadia–Hofstee graphs confirmed that these

compounds are competitive KLK7 inhibitors. Of these new inhibitors, compounds **9g** and **9j** emerged as low micromolar inhibitors of KLK7, with K_i values of 1.8 μM and 3.0 μM, respectively. The molecular dynamics simulation and MM/PB(GB)SA calculations showed good agreement with experimental results. The promising results obtained in this study make these new compounds valid leads for further optimization studies aiming to improve the potency of this new class of kallikrein inhibitors.

Acknowledgements

We gratefully acknowledge financial support from FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Proc. 11/51297-8), CNPq (Conselho Nacional de Pesquisa e Desenvolvimento, Proc. 312701/2009-8) and REUNI-CAPES for master degree scholarship.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.08.047.

References and notes

1. Diamandis, E. P.; Yousef, G. M.; Clements, J.; Ashworth, L. K.; Yoshida, S.; Egelrud, T.; Nelson, P. S.; Shiosaka, S.; Little, S.; Lilja, H.; Stenman, U.; Rittenhouse, H. G.; Wain, H. *Clin. Chem.* **2000**, *46*, 1855.
2. Harvey, T. J.; Hooper, J. D.; Myers, S. A.; Stephenson, S. A.; Ashworth, L. K.; Clements, J. A. *J. Biol. Chem.* **2000**, *275*, 37397.
3. Gan, L.; Lee, I.; Smith, R.; Argonza-Barrett, R.; Lei, H.; McCuaig, J.; Moss, P.; Paeper, B.; Wang, K. *Gene* **2000**, *257*, 119.
4. Fernández, I. S.; Ständker, L.; Mägert, H.-J.; Forssmann, W.-G.; Giménez-Gallego, G.; Romero, A. *J. Mol. Biol.* **2008**, *377*, 1488.
5. Brattsand, M.; Stefansson, K.; Lundh, C.; Haasum, Y.; Egelrud, T. *J. Invest. Dermatol.* **2005**, *124*, 198.
6. Levi, K.; Baxter, J.; Meldrum, H.; Misra, M.; Pashkovski, E.; Dauskardt, R. H. *J. Invest. Dermatol.* **2008**, *128*, 2345.
7. Ekholm, E.; Egelrud, T. *Arch. Dermatol. Res.* **1999**, *291*, 195.
8. Komatsu, N.; Saijoh, K.; Kuk, C.; Shirasaki, F.; Takehara, K.; Diamandis, E. P. *Br. J. Dermatol.* **2007**, *156*, 875.
9. Yamasaki, K.; Gallo, R. L. *J. Dermatol. Sci.* **2009**, *55*, 77.
10. Bäckman, A.; Strandén, P.; Brattsand, M.; Hansson, L.; Egelrud, T. *J. Invest. Dermatol.* **1999**, *113*, 152.
11. Komatsu, N.; Takata, M.; Otsuki, N.; Ohka, R.; Amano, O.; Takehara, K.; Saijoh, K. *J. Invest. Dermatol.* **2002**, *118*, 436.
12. Prezas, P.; Arlt, M. J. E.; Viktorov, P.; Soosaipillai, A.; Holzscheiter, L.; Schmitt, M.; Talieri, M.; Diamandis, E. P.; Krüger, A.; Magdolen, V. *Biol. Chem.* **2006**, *387*, 807.
13. Termini, L.; Maciag, P. C.; Soares, F. A.; Nonogaki, S.; Pereira, S. M. M.; Alves, V. A. F.; Longatto-Filho, A.; Villa, L. L. *Int. J. Cancer* **2010**, *127*, 485.
14. Teixeira, T. S. P.; Freitas, R. F.; Abrahão, O., Jr; Devienne, K. F.; de Souza, L. R.; Blaber, S. I.; Blaber, M.; Kondo, M. Y.; Juliano, M. A.; Juliano, L.; Puzer, L. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6112.
15. Barros, T. G.; Pinheiro, S.; Williamson, J. S.; Tanuri, A.; Pereira, H. S.; Brindeiro, R. M.; Neto, J. B. A.; Antunes, O. A. C.; Muri, E. M. F. *Synthesis* **2009**, 620.
16. Barros, T. G.; Pinheiro, S.; Williamson, J. S.; Tanuri, A.; Gomes, M., Jr; Pereira, H. S.; Brindeiro, R. M.; Neto, J. B. A.; Antunes, O. A. C.; Muri, E. M. F. *Amino Acids* **2010**, *38*, 701.
17. Muri, E. M. F.; Gomes, M., Jr; Costa, J. S.; Alencar, F. L.; Sales, A., Jr; Bastos, M. L.; Hernandez-Valdes, R.; Albuquerque, M. G.; da Cunha, E. F. F.; Alencastro, R. B.; Williamson, J. S.; Antunes, O. A. C. *Amino Acids* **2004**, *27*, 153.
18. Bencsik, J. R.; Kercher, T.; O'Sullivan, M.; Josey, J. A. *Org. Lett.* **2003**, *5*, 2727.
19. Dietrich, E.; Lubell, W. D. *J. Org. Chem.* **2003**, *68*, 6988.
20. Hirata, I.; Cezari, M. H. C.; Nakaie, C. R.; Boshcov, P.; Ito, A. S.; Juliano, M. A.; Juliano, L. *Lett. Pept. Sci.* **1994**, *1*, 299.
21. Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. *J. Med. Chem.* **2004**, *47*, 1739.
22. Kuhn, B.; Gerber, P.; Schulz-Gasch, T.; Stahl, M. *J. Med. Chem.* **2005**, *48*, 4040.
23. Thompson, D. C.; Humblet, C.; Joseph-McCarthy, D. *J. Chem. Inf. Model.* **2008**, *48*, 1081.
24. Hou, T.; Wang, J.; Li, Y.; Wang, W. *J. Chem. Inf. Model.* **2011**, *51*, 68.