Bioorganic & Medicinal Chemistry Letters 22 (2012) 7015–7018

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Prohormone-substrate peptide sequence recognition by peptidylglycine α-amidating monooxygenase and its reflection in increased glycolate inhibitor potency

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ARTICLE INFO

Article history:
Received 27 July 2012
Revised 27 September 2012
Accepted 1 October 2012
Available online 11 October 2012

Keywords:
Peptidylglycine α-amidating monooxygenase
Prohormones
Peptide substrates
Glycolate inhibitors
Human cancer

ABSTRACT

The interactions of nineteen peptide substrates and fifteen analogous peptidomimetic glycolate inhibitors with human peptidylglycine α-amidating monooxygenase (PAM) have been investigated. The substrates and inhibitors are the prohormones of calcitonin and oxytocin and their analogues. PAM both secreted into the medium by and extracted from DMS53 small lung carcinoma cells has been studied. The results show that recognition of the prooxytocin and procalcitonin peptide sequences by the enzyme extends more than four and five amino acid residues, respectively, from their C-termini. This substrate sequence recognition is mirrored by increased inhibitor potency with increased peptide length in the glycolate peptidomimetics. Substitution of the C-terminal penultimate glycine and proline residues of prooxytocin and procalcitonin and their analogues with phenylalanine increases the enzyme binding affinity. However, this changes the binding mode from one that depends on peptide sequence recognition to another primarily determined by the phenylalanine moiety, for both the substrates and analogous glycolate inhibitors.

More than half of all the bioactive peptide hormones require C-terminal amidation for full biological activity, the catalysis of which is accomplished by peptidylglycine α-amidating monooxygenase (PAM).[1] Being a bifunctional enzyme, PAM consists of two subunits: peptidylglycine α-hydroxylating monooxygenase (PHM) that initiates formation of a radical at a substrate's C-terminal glycine α-carbon to then give an α-hydroxylated glycine derivative; and peptidylamidoglycolate lyase (PAL) which catalyzes cleavage of this hydroxyglycine intermediate to give the amidated product with consequent release of glyoxylate (Scheme 1). Apart from their ubiquitous use in nature, these amidated peptides have been implicated in a number of pathological conditions such as inflammation,[2] asthma,[3] arthritis[4] and cancer.[5–12] Calcitonin, for example, is a C-terminal amidated neuropeptide that has been found to inhibit apoptosis of prostate cancer cell lines, thus promoting growth of the cancer.[5,6] Oxytocin, another amidated peptide, which normally induces contractions during labour as well as the milk let-down response during breast-feeding in mammalian species, is produced by a number of cancer cell lines[7] and is known to induce metastasis of cancer masses.[8–11] This role of C-terminal amidated peptides in pathological conditions has led to interest in drug development based on the production of inhibitors that regulate PAM activity.[13–30]

Potency and selectivity are important features of inhibitors. Potent inhibitors are required to achieve low drug doses, whilst selectivity is important for avoiding generic side effects. Micromolar glycolate inhibitors of bovine PAM were reported by May and coworkers.[13,14] We reinvestigated compounds of this class for their inability to form radical intermediates through interaction with PHM,[15] and recently reported their selective inhibition, at nanomolar concentrations, of PAM extracted from small cell lung carcinoma cells.[16] On this basis, glycolate PAM inhibitors were chosen as a focus of this study. PAM has been shown to recognise the C-terminal penultimate amino acid of peptidyl substrates.[14,17–19] Further, Tamburini et al.,[20] found that prohormones and their C-terminal pentapeptide mimics show equal binding affinity with PAM, indicating that recognition by the enzyme is based entirely on this region of the substrates. We decided to explore this further by measuring the apparent K_m values of the N-acetylated amino acid, and di-, tri- and tetra-peptide analogues **1** and **3a–c** (Fig. 1) of the C-terminus of the oxytocin prohormone (**3d**), as well as that of prooxytocin (**3d**) itself, in competitive assays with (*R*)-Tyr-(*S*)-Val-Gly, as substrates of PAM. Since it has also been shown that PAM binds preferentially to peptides that contain a phenylalanine residue at the C-terminal penultimate position,[19] the study has been extended to include the analogous phenylalanine derivatives **4a–d**. Similarly, the di-, tri-, tetra- and penta-peptide analogues **7a–d** of the C-terminus of the calcitonin prohormone (**7e**), as well as procalcitonin (**7e**) itself, and the analogous phenylalanine derivatives **8a–e** have

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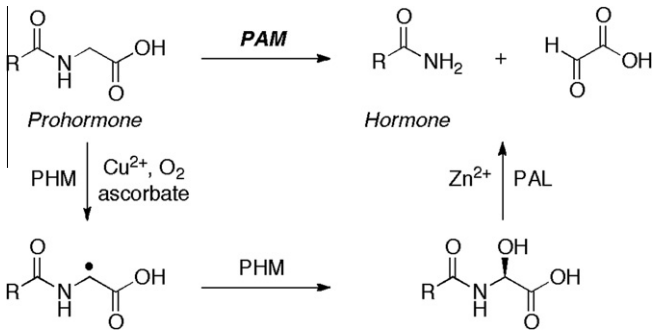
http://dx.doi.org/10.1016/j.bmcl.2012.10.004



Scheme 1. Biosynthesis of C-terminal amidated peptide hormones catalyzed by PAM.

been investigated. In addition, the corresponding glycolate peptidomimetics **2**, **5a–d**, **6a–d**, **9a–c** and **10a–c** have been studied. We[15] and others[13] have determined that compounds of this class are competitive inhibitors of PAM. We now find that the potency of inhibition of PAM by the glycolates **2**, **5a–d**, **6a–d**, **9a–c** and **10a–c** reflects the prohormone-substrate sequence recognition apparent from the results with the glycine derivatives **1**, **3a–d**, **4a–d**, **7a–e** and **8a**–**e**.

The PAM used in these experiments was cellular enzyme extracted from DMS53 small cell lung carcinoma cells (**cellular PAM**), and that secreted into the medium by those cells (**medium PAM**).[16] The DMS53 cell line is available from the American Type Culture Collection and known to produce both calcitonin and oxytocin.[7] The glycine derivatives **1**, **3a–c**, **4a–c**, **7a–d** and **8a–d** were either obtained commercially or prepared by acetylation of the corresponding peptides. The peptidomimetic glycolates **5a–c**, **6a–c**, **9a–c** and **10a–c** were synthesized by treatment of the corresponding, appropriately protected, amino acids and peptides with either benzyl bromoacetate or *tert*-butyl bromoacetate, followed by hydrogenation or hydrolysis to remove the protecting groups. The glycolate **2** was purchased. Prooxytocin (**3d**) and procalcitonin (**7e**) and the analogous phenylalanine derivatives **4d** and **8e**, as well as the corresponding glycolates **5d** and **6d**, were prepared using solid-phase peptide synthesis. Enzyme $K_{m,app}$ and IC_{50} values were determined in competitive assays using the tripeptide (*R*)-Tyr-(*S*)-Val-Gly as the substrate.[16] The results are shown in Table 1, with standard deviations from experiments performed at least in triplicate, and analysed at least in duplicate. Further details are provided as Supplementary data.

Comparison of the apparent K_m values of the N-acetylated amino acid, and di-, tri- and tetra-peptides **1** and **3a–c**, and of prooxytocin (**3d**), with the cellular PAM, shows increased binding affinity with increased peptide length ($K_{m,app}$ **1** > **3a** > **3b** > **3c** > **3d**). The value for prooxytocin (**3d**) is more than an order of magnitude less than that seen even with the acylated tetrapeptide **3c**, indicating that

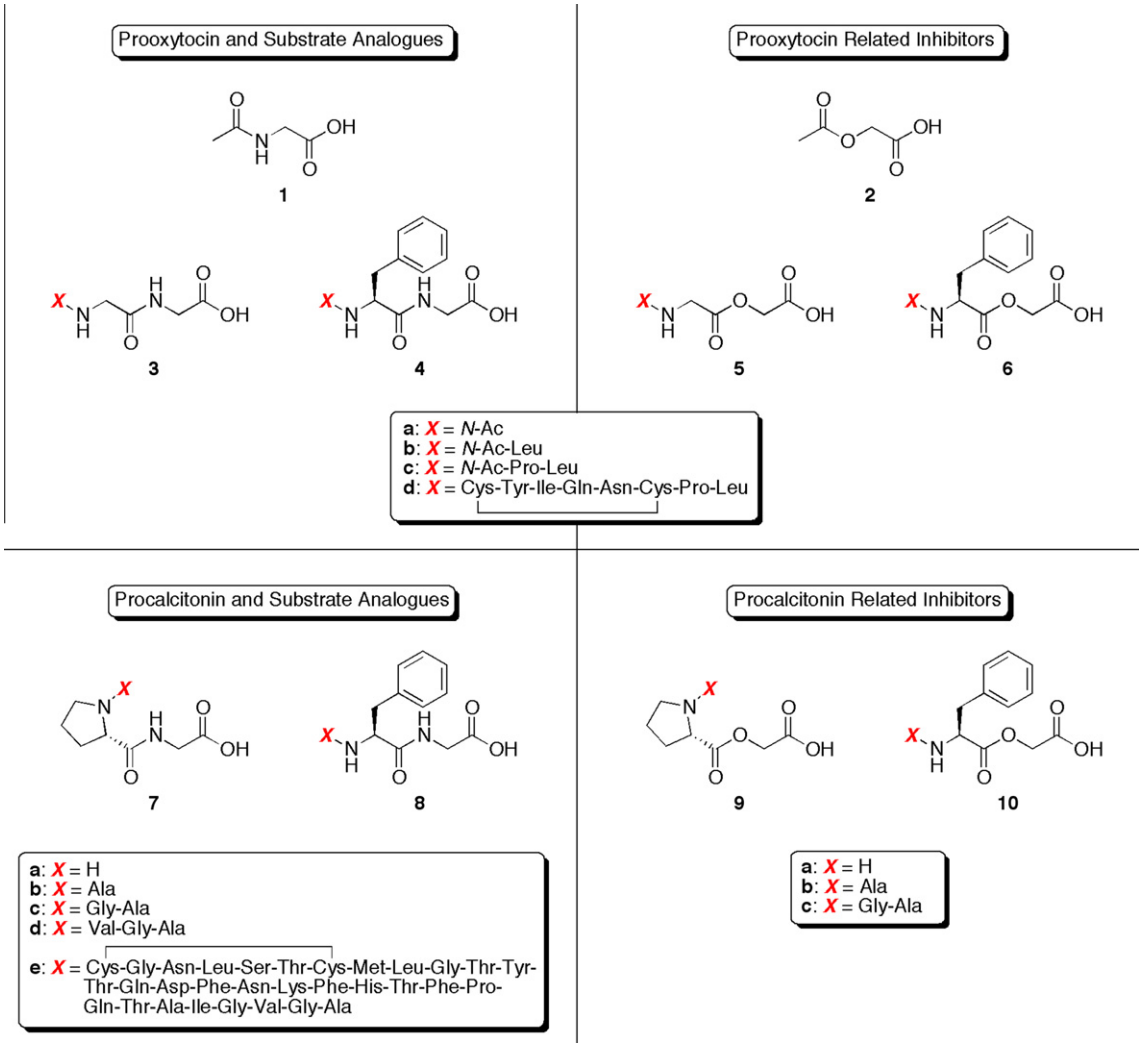


Figure 1. Substrates and inhibitors of peptidylglycine α-amidating monooxygenase used in this study. All amino acid residues have the α-(*S*)-configuration.

Table 1
$K_{m,app}$ and IC_{50} values[a] for interactions of prooxytocin (**3d**) and procalcitonin (**7e**) and related substrates and inhibitors with PAM

Substrates	$K_{m,app}$ (μM)		Inhibitors	IC_{50} (μM)		Substrates	$K_{m,app}$ (μM)		Inhibitors	IC_{50} (μM)	
	Cellular PAM	Medium PAM		Cellular PAM	Medium PAM		Cellular PAM	Medium PAM		Cellular PAM	Medium PAM
Oxytocin analogues											
1	7500 ± 500	9900 ± 2000	**2**	1100 ± 170	1100 ± 200						
3a	1600 ± 3	1200 ± 90	**5a**	210 ± 40	1800 ± 350	**4a**	6 ± 1	16 ± 3	**6a**	5 ± 0.3	11 ± 2
3b	860 ± 80	860 ± 150	**5b**	120 ± 7	200 ± 8	**4b**	8 ± 1	11 ± 0.7	**6b**	3 ± 0.6	9 ± 0.1
3c	660 ± 80	550 ± 100	**5c**	76 ± 5	180 ± 4	**4c**	6 ± 0.3	7 ± 0.2	**6c**	6 ± 0.3	38 ± 0.4
3d	36 ± 3	190 ± 8	**5d**	12 ± 1	71 ± 7	**4d**	5 ± 0.8	27 ± 2	**6d**	2 ± 0.3	23 ± 4
Calcitonin analogues											
7a	980 ± 150	880 ± 90	**9a**	600 ± 100	500 ± 30	**8a**	6000 ± 900	6500 ± 780	**10a**	1100 ± 200	1600 ± 290
7b	5000 ± 450	4000 ± 770	**9b**	370 ± 50	290 ± 40	**8b**	180 ± 5	200 ± 40	**10b**	350 ± 60	370 ± 50
7c	3000 ± 600	3000 ± 500	**9c**	980 ± 150	1100 ± 170	**8c**	40 ± 5	35 ± 7	**10c**	560 ± 50	160 ± 4
7d	1000 ± 100	960 ± 180				8d	8 ± 0.2	18 ± 3			
7e	200 ± 25	200 ± 40				8e	8 ± 0.2	14 ± 4			

[a] Due to the limitations of comparing $K_{m,app}$ and IC_{50} values, and in interpreting $K_{m,app}$ values as a measure of binding affinity, conclusions drawn in this manuscript are based on patterns observed independently in the $K_{m,app}$ values of the substrates **1**, **3a–d**, **4a–d**, **7a–e** and **8a–e** and the IC_{50} values of the inhibitors **2**, **5a–d**, **6a–d**, **9a–c** and **10a–c**.

substrate recognition extends more than four amino acid residues from the C-terminus. These findings are consistent with earlier observations[31] that 'relatively short model substrates cannot mimic that full range of enzyme-substrate contacts that exist in the PHM/PAM active site for longer peptide substrates'. An identical trend of peptide sequence recognition is observed with compounds **1** and **3a–c** and the medium PAM. The peptidomimetic glycolate prooxytocin analogues **2** and **5a–d** display this same pattern with the cellular PAM (IC **2** > **5a** > **5b** > **5c** > **5d**), showing that their potency as PAM inhibitors follows the relationship of amino acid sequence recognition apparent from the data obtained for the corresponding substrates **1** and **3a–d**. Analogous behaviour is observed with the glycolates **2** and **5a–d** and the medium PAM. This correlation holds even though, in common with the results of earlier studies and their interpretation,[16] the glycolates **5a–d** are more potent inhibitors of the cellular PAM than of the enzyme secreted into the medium, and they also show greater binding affinity than the corresponding substrates **3a–d** for the cellular protein. Further, the glycolates **5a–d** selectively inhibit the cellular PAM, compared to the enzyme in the medium, whereas the substrates **1** and **3a–d** do not exhibit this behaviour pattern.

The prooxytocin-related substrates **4a–d** having phenylalanine instead of glycine as the C-terminal penultimate amino acid residue do not show the sequence recognition seen with the glycine derivatives **1** and **3a–d**. They all bind to the cellular PAM with $K_{m,app}$ values between 5–8 μM, and show similar but slightly reduced binding affinities for the medium PAM. The high binding affinities of the phenylalanine derivatives **4a–d** are consistent with the earlier report of the preferential binding of such species to PAM.[19] However, the contrast between the relative lack of impact of the N-terminal portion of these compounds **4a–d** on the $K_{m,app}$ values and the sequence recognition displayed by the glycine derivatives **1** and **3a–d** indicates that the two groups of compounds adopt different binding modes, dominated by the phenylalanine portion and peptide sequence, respectively.

The phenylalanylglycolates **6a–d** are all low micromolar inhibitors of PAM with binding affinities very similar to those of the analogous substrates **4a–d**. Combined with the lack of any effect of structural differences between the glycolates **6a–d** on the IC_{50} values, this indicates that, in common with the substrates **4a–d**, the strength of their association with the enzyme is primarily determined by the interactions with the phenylalanine portion. The inhibitors **6a–d** do not exhibit peptide sequence recognition but, nevertheless, their affinity for the enzyme closely parallels that of the corresponding substrates **4a–d**.

Differences between the trends apparent for the prooxytocin-related substrates **1**, **3a–d** and **4a–d**, and inhibitors **2**, **5a–d** and **6a–d**, and the results obtained for the corresponding procalcitonin-related compounds **7a–e**, **8a–e**, **9a–c** and **10a–c** are most likely due to the impact of the interactions of the N-terminal free amino group of the latter compounds with PAM. Whereas the N-acetylated analogues **1**, **2**, **3a–c**, **4a–c**, **5a–c** and **6a–c** of prooxytocin (**3d**) retain an amide bond at the N-terminus in place of a peptide bond of prooxytocin (**3d**), in place of a peptide bond of procalcitonin (**7e**) the procalcitonin analogues **7a–d**, **8a–d**, **9a–c** and **10a–c** instead have an amino group, that will be protonated under the assay conditions. This is likely to have the greatest impact on the binding affinities of the shorter compounds **7a,b**, **8a,b**, **9a,b** and **10a,b**. Even so, the $K_{m,app}$ values of the tetra- and penta-peptides **7c** and **7d**, and of procalcitonin (**7e**), with both the cellular and the medium PAM, show increased binding affinity with increased peptide length ($K_{m,app}$ **7c** > **7d** > **7e**), with the value for procalcitonin (**7e**) being about one fifth of that seen with the pentapeptide **7d** with PAM from either source. This shows that recognition of the substrate calcitonin (**7e**) extends more than five amino acid residues from the C-terminus, even further than previously thought.[19]

If considered in isolation, the pattern of increased binding affinity with increased peptide length observed with the procalcitonin-related phenylalanine derivatives **8a–e** could be interpreted to be the consequence of recognition of the lengthening peptide sequence. However, in the context of the widely different $K_{m,app}$ values of the substrates **4a** and **8a**, and the IC_{50} values of the inhibitors **6a** and **10a**, it is clear that this is not the case. The binding affinities of the free amines **8a** and **10a** are much less than those of the corresponding N-acetylated but otherwise identical compounds **4a** and **6a**, showing that the protonated amino groups of the amines **8a** and **10a** cause their relatively weak binding. Comparison of the $K_{m,app}$ values shows that there is no recognition of the extended peptide sequence with the phenylalanine derivatives **4a** and **8c–e**. Instead, the close similarity between the data for the procalcitonin-related species **8c–e** and the prooxytocin analogues **4a–d** appears to be a consequence of all these compounds binding to PAM in a similar way, determined in each case mainly by recognition of the phenylalanine component.

Given the complexity associated with the N-terminal amino groups of the peptidylglycolates **9a–c** and **10a–c**, it is not feasible to draw any general conclusions from the potency of these compounds as PAM inhibitors. Nevertheless, overall the results of this study show that peptide sequence recognition is an important aspect of the interactions of the prohormone substrates prooxytocin

(**3d**) and procalcitonin (**7e**) with PAM, which is mirrored in the potency of analogous peptidomimetic glycolate inhibitors of the enzyme. Substitution of glycine or proline with phenylalanine at the C-terminal penultimate position of substrates and inhibitors increases the enzyme binding affinity, which is then independent of the peptide sequence. It is particularly noteworthy that this applies with the natural substrates of PAM, prooxytocin (**3d**) and procalcitonin (**7e**), where replacement of the C-terminal penultimate amino acid with phenylalanine increases the binding affinity of the analogues **4d** and **8e** by around an order of magnitude, and the corresponding phenylalanylglycolate inhibitors **5d** and **6d** then retain this potency. That is, the features can be exploited in the design of inhibitors that bind selectively to the enzyme, with a potency that surpasses that of their analogous substrates.

Acknowledgments

We gratefully acknowledge the support received for this work from the Australian Research Council, and the assistance of Dr. M. J. Gresser and Mr. J. Kerr with glycolate syntheses and Dr. H.-K. Kim with culturing DMS53 cells.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.004.

References and notes

1. Prigge, S. T.; Mains, R. E.; Eipper, B. A.; Amzel, L. M. *Cell. Mol. Life Sci.* **2000**, *57*, 1236.
2. Wiesenfeld-Hallin, Z.; Xu, X.-J. *Eur. J. Pharmacol.* **2001**, *429*, 49.
3. Groneberg, D. A.; Springer, J.; Fischer, A. *Pulm. Pharmacol. Ther.* **2001**, *14*, 391.
4. Levine, J. D.; Clark, R.; Devor, M.; Helms, C.; Moskowitz, M. A.; Basbaum, A. I. *Science* **1984**, *226*, 547.
5. Chien, J.; Ren, Y.; Wang, Y. Q.; Bordelon, W.; Thompson, E.; Davis, R.; Rayford, W.; Shah, G. *Mol. Cell. Endocrinol.* **2001**, *181*, 69.
6. Salido, M.; Vilches, J.; López, A.; Roomans, G. M. *Endocr. Relat. Cancer* **2002**, *94*, 368.
7. Sorenson, G. D.; Pettengill, O. S.; Brinck-Johnsen, T.; Cate, C. C.; Maurer, L. H. *Endocr. Relat. Cancer* **1981**, *47*, 1289.
8. Péqueux, C.; Breton, C.; Hendrick, J.-C.; Hagelstein, M.-T.; Martens, H.; Winkler, R.; Geenen, V.; Legros, J.-J. *Cancer Res.* **2002**, *62*, 4623.
9. Péqueux, C.; Keegan, B. P.; Hagelstein, M.-T.; Geenen, V.; Legros, J.-J.; North, W. G. *Endocr. Relat. Cancer* **2004**, *11*, 871.
10. Cassoni, P.; Marrocco, T.; Bussolati, B.; Allia, E.; Munaron, L.; Sapino, A.; Bussolati, G. *Mol. Cancer Res.* **2006**, *4*, 351.
11. Zhong, M.; Boseman, M. L.; Millena, A. C.; Khan, S. A. *Mol. Cancer Res.* **2010**, *8*, 1164.
12. Schally, A. V.; Comaru-Schally, A. M.; Nagy, A.; Kovacs, M.; Szepeshazi, K.; Plonowski, A.; Varga, J. L.; Halmos, G. *Front. Neuroendocrinol.* **2001**, *22*, 248.
13. Katopodis, A. G.; May, S. W. *Biochemistry* **1990**, *29*, 4541.
14. Ping, D.; Mounier, C. E.; May, S. W. *J. Biol. Chem.* **1995**, *270*, 29250.
15. Barratt, B. J. W.; Easton, C. J.; Henry, D. J.; Li, I. H. W.; Radom, L.; Simpson, J. S. *J. Am. Chem. Soc.* **2004**, *126*, 13306.
16. Cao, F.; Gamble, A. B.; Kim, H.-K.; Onagi, H.; Gresser, M. J.; Kerr, J.; Easton, C. J. *MedChemCommun* **2011**, *2*, 760.
17. Bradbury, A. F.; Smyth, D. G. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 372.
18. Murthy, A. S. N.; Keutmann, H. T.; Eipper, B. A. *Mol. Endocrinol.* **1987**, *1*, 290.
19. Tamburini, P. P.; Young, S. D.; Jones, B. N.; Palmesino, R. A.; Consalvo, A. P. *Int. J. Pept. Protein Res.* **1990**, *35*, 153.
20. Tamburini, P. P.; Jones, B. N.; Consalvo, A. P.; Young, S. D.; Lovato, S. J.; Gilligan, J. P.; Wennogle, L. P.; Erion, M.; Jeng, A. Y. *Arch. Biochem. Biophys.* **1988**, *267*, 623.
21. Ogonowski, A. A.; May, S. W.; Moore, A. B.; Barrett, L. T.; O'Bryant, C. L.; Pollock, S. H. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 846.
22. Andrews, M. D.; O'Callaghan, K. A.; Vederas, J. C. *Tetrahedron* **1997**, *53*, 8295.
23. Bolkenius, F. N.; Ganzhorn, A. J. *Gen. Pharmacol.* **1998**, *31*, 655.
24. Mueller, G. P.; Driscoll, W. J.; Eipper, B. A. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 1331.
25. Kulathila, R.; Merkler, K. A.; Merkler, D. J. *Nat. Prod. Rep.* **1999**, *16*, 145.
26. Feng, J.; Shi, J.; Sirimanne, S. R.; Mounier-Lee, C. E.; May, S. W. *Biochem. J.* **2000**, *350*, 521.
27. Trendel, J. A.; Ellis, N.; Sarver, J. G.; Klis, W. A.; Dhanajeyan, M.; Bykowski, C. A.; Reese, M. D.; Erhardt, P. W. *J. Biomol. Screen.* **2008**, *13*, 804.
28. Merkler, D. J.; Asser, A. S.; Baumgart, L. E.; Carballo, N.; Carpenter, S. E.; Chew, G. H.; Cosner, C. C.; Dusi, J.; Galloway, L. C.; Lowe, A. B.; Lowe, E. W., Jr.; King, L., III; Kendig, R. D.; Kline, P. C.; Malka, R.; Merkler, K. A.; McIntyre, N. R.; Romero, M.; Wilcox, B. J.; Owen, T. C. *Bioorg. Med. Chem.* **2008**, *16*, 10061.
29. Schade, D.; Kotthaus, J.; Hungeling, H.; Kotthaus, J.; Clement, B. *ChemMedChem* **2009**, *4*, 1595.
30. Langella, E.; Pierre, S.; Ghattas, W.; Giorgi, M.; Réglier, M.; Saviano, M.; Esposito, L.; Hardré, R. *ChemMedChem* **2010**, *5*, 1568.
31. Chew, G. H.; Galloway, L. C.; McIntyre, N. R.; Schroder, L. A.; Richards, K. M.; Miller, S. A.; Wright, D. W.; Merkler, D. J. *FEBS Lett.* **2005**, *579*, 4678.