Bioorganic & Medicinal Chemistry Letters 22 (2012) 2555–2559

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Penicillin inhibitors of purple acid phosphatase

Faridoon [a,b], Waleed M. Hussein [a,c], Nazar Ul Islam [b], Luke W. Guddat [a], Gerhard Schenk [a,d], Ross P. McGeary [a,e,*]

[a] The University of Queensland, School of Chemistry and Molecular Biosciences, Brisbane, Qld 4072, Australia
[b] Institute of Chemical Sciences, University of Peshawar, Peshawar-25120, Pakistan
[c] Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Helwan University, Ein Helwan, Egypt
[d] National University of Ireland-Maynooth, Department of Chemistry, Maynooth, Co. Kildare, Ireland
[e] The University of Queensland, School of Pharmacy, Brisbane, Qld 4072, Australia

ARTICLE INFO

Article history:
Received 4 January 2012
Revised 26 January 2012
Accepted 30 January 2012
Available online 4 February 2012

Keywords:
Purple acid phosphatase (PAP)
Osteoporosis
Penicillin
Inhibition assays

ABSTRACT

Purple acid phosphatases (PAPs) are binuclear metallohydrolases that have a multitude of biological functions and are found in fungi, bacteria, plants and animals. In mammals, PAP activity is linked with bone resorption and over-expression can lead to bone disorders such as osteoporosis. PAP is therefore an attractive target for the development of drugs to treat this disease. A series of penicillin conjugates, in which 6-aminopenicillanic acid was acylated with aromatic acid chlorides, has been prepared and assayed against pig PAP. The binding mode of most of these conjugates is purely competitive, and some members of this class have potencies comparable to the best PAP inhibitors yet reported. The structurally related penicillin G was shown to be neither an inhibitor nor a substrate for pig PAP. Molecular modelling has been used to examine the binding modes of these compounds in the active site of the enzyme and to rationalise their activities.

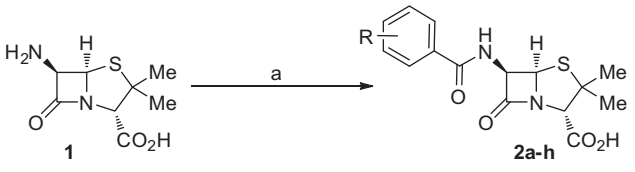
Purple acid phosphatases (PAPs)[1] are binuclear metallohydrolases which are associated with disorders such as osteoporosis,[2–4] hairy cell leukaemia,[5] Gaucher disease,[6] and AIDS.[7] These enzymes have a characteristic purple colour due to a charge transfer transition between a tyrosine phenolate and Fe(III) in the active site.[8] PAPs catalyse the hydrolysis of various phosphorylated substrates at neutral to acidic pH values according to the equation:[1]

$$RO\text{-}PO_3^{2-} + H_2O \rightarrow ROH + HPO_4^{2-}$$

Mammalian PAPs are monomeric with a molecular mass of 35 kDa and contain a redox active Fe(III)–Fe(III/II) centre in their active site.[9,10] Plant PAPs are homodimeric with a molecular mass of 110 kDa and a redox inactive Fe(III)–Zn(II) or Fe(III)–Mn(II) centre.[1,11–13] The mammalian enzyme is secreted by osteoclasts[14] into the bone-resorptive space, where it is required for normal bone resorption.[15] Osteoporosis, a decrease in bone mineral density, occurs when the rate of bone resorption exceeds that of bone formation, creating an imbalance in the dynamic bone remodelling process.[16] The association of elevated PAP levels in the serum with the onset of osteoporosis[14,17–19] has prompted us to develop potent inhibitors of this enzyme.[20–22] Whilst clinical treatments are available for osteoporosis, particularly bisphosphonates which inhibit farnesyl pyrophosphate synthase in osteoclasts,[23,24] these drugs have significant side effects and compliance issues.

Many small anions including fluoride[25–28] and tetrahedral oxoanions such as phosphate, arsenate, vanadates,[29] tungstate and molybdate[30,31] have been reported as weak PAP inhibitors. Simple phosphonate-containing molecules with pendant metal binding groups such as carboxylate and thiol,[32] and several modified phosphotyrosine-containing tripeptides[33] are also PAP inhibitors. Recently, our group reported a series of α-alkoxynaphthylmethyl phosphonic acids which inhibit pig PAP (pPAP) with K_i values as low as 17 μM, and inhibit red kidney bean PAP (rkbPAP) with K_i values as low as 4 μM.[20] We have also reported the activities of structurally related compounds, acylated α-aminonaphthylmethylphos phonic acids, which have K_i values as low as 8 μM against pPAP and 5 μM against rkbPAP.[22]

We report here our efforts at identifying potent inhibitors of pPAP by exploring the inhibitory effects of penicillin conjugates.

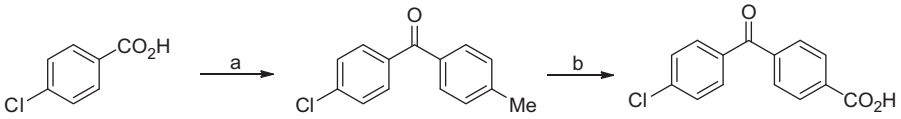


a, R = 4-(PhCO); **b**, R = 4-(2-ClC$_6$H$_4$CO); **c**, R = 4-(4-ClC$_6$H$_4$CO); **d**, R = 4-(3-NO$_2$C$_6$H$_4$CO); **e**, R = 4-(4-NO$_2$C$_6$H$_4$CO); **f**, R = 3-(4-MeC$_6$H$_4$CO); **g**, R = 3-(piperidinylcarbonyl); **h**, R = 4-OAc

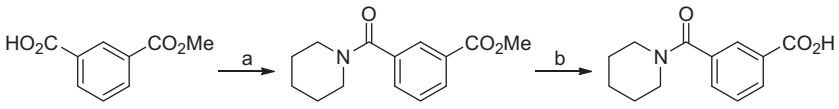
Scheme 1. Reagents and conditions: (a) ArCOCl, 2% aq $NaHCO_3$, 0 °C, acetone, 2–4 h 71% (**2a**); 65% (**2b**); 51% (**2c**); 60% (**2d**); 66% (**2e**); 53% (**2f**); 51% (**2g**); 53% (**2h**).

* Corresponding author. Tel.: +61 7 3365 3955; fax: +61 7 3346 3249.
E-mail address: r.mcgeary@uq.edu.au (R.P. McGeary).

0960-894X/$ - see front matter Crown Copyright © 2012 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2012.01.123



Scheme 2. Synthesis of the aryl carboxylic acid used for the preparation of penicillin conjugate **2c**. Reagents and conditions: (a) PhMe, P_2O_5–SiO_2, Δ (49%); (b) Jones reagent, AcOH, Δ, 4 h (35%).



Scheme 3. Synthesis of the aryl carboxylic acid used for the preparation of penicillin conjugate **2g**. Reagents and conditions: (a) Me_3COCl, Et_3N, piperidine, CH_2Cl_2, rt, 18 h (78%); (b) $LiOH·H_2O$, MeOH–H_2O, rt, 18 h (80%).

Merck scientists pioneered the investigation of β-lactams as potential therapeutics other than for their common use as antibiotics. β-Lactams have been identified as *irreversible* inactivators of many classes of serine and cysteine proteases, including porcine pancreatic elastase, human neutrophil elastase, *Escherichia coli* signal peptidase, prostate-specific antigen, cathepsin G, thrombin, chymotrypsin, trypsin, plasmin, human cytomegalovirus protease, poliovirus and human rhinovirus 3C proteases, and papain.[34] The application of β-lactams as *reversible* enzyme inhibitors has been little explored, although reversible β-lactam inhibitors of human leucocyte elastase and porcine pancreatic elastase,[35] carboxypeptidase A,[36] and galactosidase[37] have been reported. The structural and functional similarities between PAPs and the β-lactam-degrading metallo-β-lactamases (both enzymes are binuclear metallohydrolases[38]) prompted us to examine whether suitably functionalised β-lactam antibiotics might inhibit the activity of PAP. Here, we disclose that penicillin conjugates (**2a–h**) are good inhibitors of pPAP, comparable in potency to the most effective PAP inhibitors reported to date.

The conjugates **2a–h** were prepared by acylation of the sensitive 6-aminopenicillanic acid (6-APA) **1** with different aromatic acid chlorides in a mixture of aqueous sodium bicarbonate solution and acetone (Scheme 1). Compound **2i** is commercially available penicillin G.

Compound **2a** was prepared using commercially available 4-benzoylbenzoic acid. The syntheses of the aromatic carboxylic acids required to prepare compounds **2b** and **2d–f** have been reported by us elsewhere.[39] The preparation of the aromatic carboxylic acid required for the synthesis of compound **2c** is shown in Scheme 2, and the synthesis of the carboxylic acid used in the preparation of compound **2g** is shown in Scheme 3. 4-Acetoxybenzoic acid, required for the synthesis of compound **2h**, was prepared by acetylation of 4-hydroxybenzoic acid[40] (see Supplementary data).

The inhibitory effects of the penicillin conjugates **2a–i** were tested against pPAP at pH 4.9 using a standard spectrophotometric assay, with *para*-nitrophenyl phosphate as substrate, as described elsewhere.[20,22,25–29] The determined competitive (K_{ic}) and uncompetitive (K_{iuc}) inhibition constants are listed in Table 1.

For inhibitors **2a–f,h**, only competitive inhibition was observed against pPAP. We found that penicillin conjugates with *para*-substituted 4-benzoylbenzoic acid side chains (**2c** and **2e**) are the most potent, with K_{ic} values of 12 μM and 17 μM, respectively. To assess the effect of a nitrogen heterocyclic ring on the inhibitory activity, we replaced the terminal aromatic ring of **2f** with piperidine in compound **2g**. However, the kinetic data showed that **2g** has reduced inhibitory potency and, unlike the other inhibitors listed in Table 1, both competitive and uncompetitive inhibition are observed. Another intriguing result shown in Table 1 is that compound **2i**, penicillin G, has no inhibitory effect on pPAP. This is surprising, given the close structural similarity between compound **2i** and the inhibitory compounds listed in Table 1.

Table 1
Kinetic data for inhibitors against pPAP at pH 4.9[a]

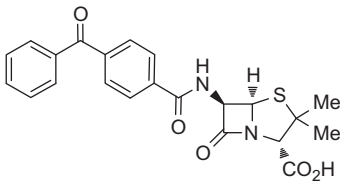
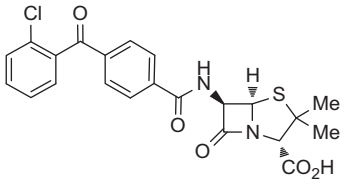
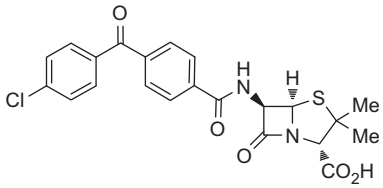
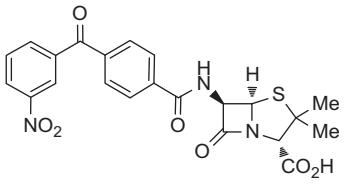
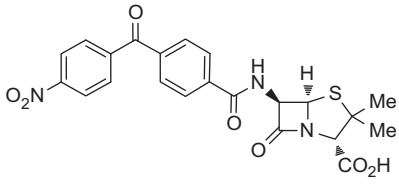
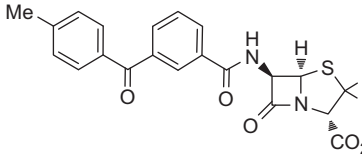
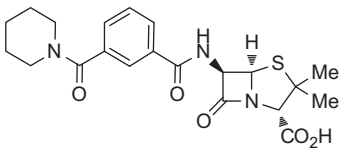
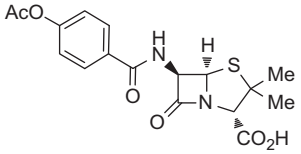
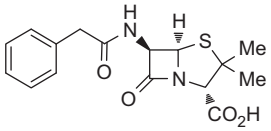
Inhibitors		K_{ic}[b] (μM)	K_{iuc}[c] (μM)
	**2a**	26 ± 12	–[d]
	**2b**	32 ± 8	–
	**2c**	12 ± 4	–
	**2d**	23 ± 8	–
	**2e**	17 ± 7	–
	**2f**	60 ± 59	–

Table 1 (*continued*)

Inhibitors		K_{ic}[b] (μM)	K_{iuc}[c] (μM)
	**2g**	241 ± 207	491 ± 558
	**2h**	53 ± 17	–
	**2i**	–	–

[a] Values ± standard errors of at least two replicates—see Supplementary data.
[b] K_{ic}, competitive inhibition constant.
[c] K_{iuc}, uncompetitive inhibition constant.
[d] No significant effect.

To investigate the possible binding modes of the most potent inhibitor, **2c**, to pPAP, in silico docking was employed using Molegro Virtual Docker.[41] The pPAP crystal structure (PDB Code: 1UTE)[42] was used in this study. To prepare the crystal structure for docking, the Molegro Virtual Docker was used to define the active site, set charges of +3 for Fe1 and +2 for Fe2, and to assign Phe 56, Gln 151 and Phe 244 as flexible residues. PyMol was used to visualise the final docking results.

The lowest energy binding orientation of **2c** in the active site of pPAP is shown in Figure 1. Modelling suggests that **2c** binds close to the two metal ions via its carboxylate group, with one oxygen bridging the metal ions (with O–M distances of 2.0 Å (Fe1) and 3.3 Å (Fe2)) and the other oxygen atom of the carboxylate group in **2c** binding weakly to the two metal ions (with O–M distances of 4.0 Å (Fe1) and 3.3 Å (Fe2)). In addition, the oxygen of the carbonyl group in the β-lactam ring of **2c** forms a hydrogen bond to the NH group of the imidazole side chain of His 223 (N–O distance 2.6 Å) and there is another hydrogen bond between the nitrogen atom of the β-lactam ring and the NH group of the imidazole side chain of His 195 (N–N distance 3.2 Å). There is also a hydrophobic interaction between the benzyl group of Phe 244 and the chlorobenzene moiety of **2c**. Importantly, modelling predicts a favourable interaction between the negatively charged carboxylate side-chain of Asp 246

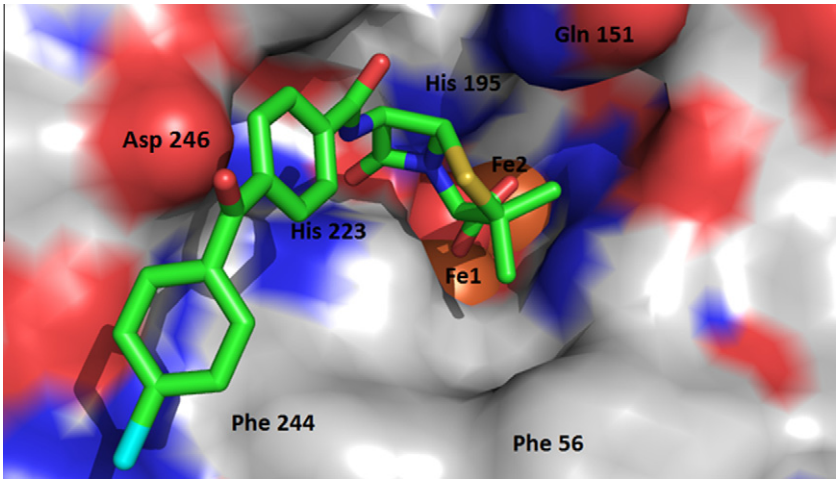


Figure 1. Surface view of the enzyme active site for the highest Molegro Virtual Docker score conformation of pPAP with **2c** docked into the active site. Atom colours are as follows: blue—nitrogen, red—oxygen, white—carbon (on pPAP), sulfur—yellow, green—carbon, cyan—chlorine (on inhibitor), orange—iron active site metals.

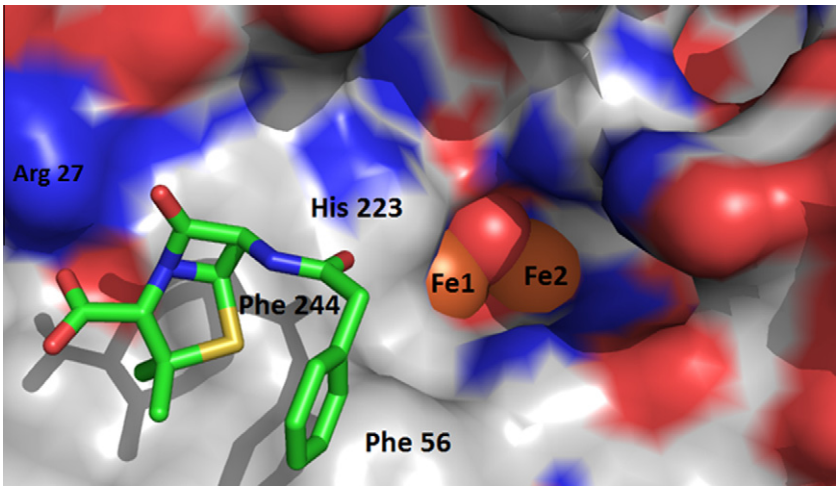


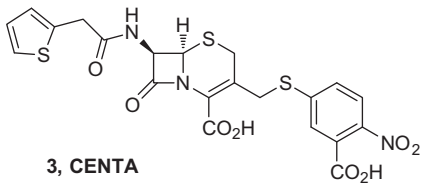
Figure 2. Surface view of the enzyme active site for the highest Molegro Virtual Docker score conformation of pPAP with penicillin G (**2i**) docked into the active site. Atom colours are as follows: blue—nitrogen, red—oxygen, white—carbon (on pPAP), sulfur—yellow, green—carbon, orange—iron active site metals.

and the partial positive charge on the biaryl carbonyl carbon; the distance between this carbon atom and the oxygen atom of the Asp 246 carboxylate group is 4.8 Å.

The proposed binding mode of **2c** within the active site of pPAP through the carboxylate group differs from the mode of binding of β-lactam antibiotics in the active site of the structurally related metallo-β-lactamases. In these structures the oxygen atom of the carbonyl group of the β-lactam is believed to bind to one of the two zinc ions, activating it for nucleophilic attack by a proximal metal-bridging hydroxide.[43] The modelling shown in Figure 1 is therefore consistent with our findings that inhibitor **2c** is competitive, and is not a substrate for pPAP.

Modelling also led to some suggestions as to why penicillin G (**2i**) is not an inhibitor of pPAP. Figure 2 shows the lowest energy binding orientation of **2i** with pPAP. It can be seen that **2i** does not bind in the active site of pPAP, preferring a stabilising electrostatic interaction between the side chain of Arg 27 and the carboxylate group of the **2i** (N–O distance 3.5 Å); the β-lactam carbonyl oxygen also interacts with the Arg 27 side chain. This leaves the active site of pPAP exposed. As noted above, we suggest that a contributing factor for the potency of inhibitor **2c** is the favourable interaction between the biaryl carbonyl group and the side chain of Asp 246 (Fig. 1). Penicillin G (**2i**) lacks this carbonyl group, and so cannot interact in this way with the side chain of Asp 246. It is notable that the most potent of our inhibitors listed in Table 1 bear the same 1,4-dicarbonylbenzene moiety.

Again this modelling is consistent with our observations that **2i** is not an inhibitor, nor was it a substrate for pPAP under our assay conditions. The stability of β-lactams towards pPAP was further confirmed using the metallo-β-lactamase substrate, cephem CENTA **3**. This compound generates the intensely yellow 3-carboxy-4-nitrobenzenethiolate anion on hydrolysis of its β-lactam ring. No such colour developed when CENTA was incubated with pPAP under our assay conditions, confirming that this compound is not a substrate for pPAP.



3, CENTA

The modelling of **2c** further suggests that improvements in binding might be achieved by increasing the size of the terminal aryl ketone functionalities of these inhibitors, to exploit the surface hydrophobic patch of Phe 244 (Fig. 1). Our previous work on phosphonic acid pPAP inhibitors demonstrated that increased inhibitor potency could be achieved by incorporating into these molecules hydrophobic naphthalenes to maximise interactions with surface hydrophobic patches on the enzyme.[22] Efforts are underway to co-crystallise inhibitor **2c** with pPAP to validate our modelling results.

In summary, 6-aminopenicillanic acid has been coupled with a number of aromatic carboxylic acids to generate a novel class of PAP inhibitors. Compound **2c** was the most active of the inhibitors examined, with a K_{ic} value of 12 μM, comparable to the most potent PAP inhibitors reported. In silico docking was used to examine the likely binding orientation of **2c** in the active site of pPAP as well as that of penicillin G (**2i**), a non-inhibitor of pPAP.

Acknowledgments

This work was supported by an Australian Research Council Grant (DP0986292). Mr. Faridoon is the recipient of a scholarship from the Pakistan Higher Education Commission (International Research Support Initiative Program).

Supplementary data

Supplementary data (experimental procedures for the preparation of compounds **2a–h** and details of the kinetic assayed performed) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.123.

References and notes

1. Mitić, N.; Smith, S. J.; Neves, A.; Guddat, L. W.; Gahan, L. R.; Schenk, G. *Chem. Rev.* **2006**, *106*, 3338.
2. Andersson, G.; Ek-Rylander, B.; Hollberg, K.; Ljusberg-Sjoelander, J.; Lang, P.; Norgard, M.; Wang, Y.; Zhang, S.-J. *J. Bone Miner. Res.* **2003**, *18*, 1912.
3. Angel, N. Z.; Walsh, N.; Forwood, M. R.; Ostrowski, M. C.; Cassady, A. I.; Hume, D. A. *J. Bone Miner. Res.* **2000**, *15*, 103.
4. Hayman, A. R.; Jones, S. J.; Boyde, A.; Foster, D.; Colledge, W. H.; Carlton, M. B.; Evans, M. J.; Cox, T. M. *Development* **1996**, *122*, 3151.
5. Hoyer, J. D.; Li, C. Y.; Yam, L. T.; Hanson, C. A.; Kurtin, P. J. *Am. J. Clin. Pathol.* **1997**, *108*, 308.
6. Schindelmeiser, J.; Radzun, H. J.; Munstermann, D. *Pathol. Res. Pract.* **1991**, *187*, 209.
7. Schindelmeiser, J.; Gullotta, F.; Munstermann, D. *Pathol. Res. Pract.* **1989**, *185*, 184.
8. Twitchett, M. B.; Sykes, A. G. *Eur. J. Inorg. Chem.* **1999**, 2105.
9. Wang, D. L.; Holz, R. C.; David, S. S.; Que, L., Jr.; Stankovich, M. T. *Biochemistry* **1991**, *30*, 8187.
10. Bernhardt, P. V.; Schenk, G.; Wilson, G. J. *Biochemistry* **2004**, *43*, 10387.
11. Beck, J. L.; McConachie, L. A.; Summors, A. C.; Arnold, W. N.; de Jersey, J.; Zerner, B. *Biochim. Biophys. Acta* **1986**, *869*, 61.
12. Durmus, A.; Eicken, C.; Sift, B. H.; Kratel, A.; Kappl, R.; Huttermann, J.; Krebs, B. *Eur. J. Biochem.* **1999**, *260*, 709.
13. Schenk, G.; Ge, Y.; Carrington, L. E.; Wynne, C. J.; Searle, I. R.; Carroll, B. J.; Hamilton, S.; de Jersey, J. *Arch. Biochem. Biophys.* **1999**, *370*, 183.
14. Oddie, G. W.; Schenk, G.; Angel, N. Z.; Walsh, N.; Guddat, L. W.; de Jersey, J.; Cassady, A. I.; Hamilton, S. E.; Hume, D. A. *Bone* **2000**, *27*, 575.
15. Susi, F. R.; Goldhaber, P.; Jennings, J. M. *Am. J. Physiol.* **1966**, *211*, 959.
16. Bonnick, S. L.; Shulman, L. *Am. J. Med.* **2006**, *119*, 25S.
17. Mauck, K. F.; Clarke, B. L. *Mayo Clin. Proc.* **2006**, *81*, 662.
18. Boonen, S. *Bone* **2007**, *40*, S26.
19. Halleen, J. M.; Tiitinen, S. L.; Ylipahkala, H.; Fagerlund, K. M.; Väänänen, H. K. *Clin. Lab.* **2006**, *52*, 499.
20. McGeary, R. P.; Vella, P.; Mak, J. Y. W.; Guddat, L. W.; Schenk, G. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 163.
21. Vella, P.; McGeary, R. P.; Gahan, L. R.; Schenk, G. *Curr. Enzyme Inhib.* **2010**, *6*, 118.
22. Mohd-Pahmi, S. H.; Hussein, W. M.; Schenk, G.; McGeary, R. P. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3092.
23. Reszka, A. A.; Rodan, G. A. *Curr. Rheumatol. Rep.* **2003**, *5*, 65.
24. Drake, M. T.; Clarke, B. L.; Khosla, S. *Mayo Clin. Proc.* **2008**, *83*, 1032.
25. Mitić, N.; Valizadeh, M.; Leung, E. W. W.; de Jersey, J.; Hamilton, S.; Hume, D. A.; Cassady, A. I.; Schenk, G. *Arch. Biochem. Biophys.* **2005**, *439*, 154.
26. Elliott, T. W.; Mitić, N.; Gahan, L. R.; Guddat, L. W.; Schenk, G. *J. Braz. Chem. Soc.* **2006**, *17*, 1558.
27. Pinkse, M. W. H.; Merkx, M.; Averill, B. A. *Biochemistry* **1999**, *38*, 9926.
28. Wang, X.; Ho, R. Y. N.; Whiting, A. K.; Que, L., Jr. *J. Am. Chem. Soc.* **1999**, *121*, 9235.
29. Crans, D. C.; Simone, C. M.; Holz, R. C.; Que, L. *Biochemistry* **1992**, *31*, 11731.
30. Zaidi, M.; Moonga, B.; Moss, D. W.; Macintyre, I. *Biochem. Biophys. Res. Commun.* **1989**, *159*, 68.
31. Crowder, M. W.; Vincent, J. B.; Averill, B. A. *Biochemistry* **1992**, *31*, 9603.
32. Myers, J. K.; Antonelli, S. M.; Widlanski, T. S. *J. Am. Chem. Soc.* **1997**, *119*, 3163.
33. Valizadeh, M.; Schenk, G.; Nash, K.; Oddie, G. W.; Guddat, L. W.; Hume, D. A.; de Jersey, J.; Burke, T. R.; Hamilton, S. *Arch. Biochem. Biophys.* **2004**, *424*, 154.
34. Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639.
35. Gérard, S.; Galleni, M.; Dive, G.; Marchand-Brynaert, J. *Bioorg. Med. Chem.* **2004**, *12*, 129.
36. Kim, D. H.; Li, Z.-H.; Lee, S. S.; Kim, K. R.; Chung, S. J.; Kim, E.-J. *Pure Appl. Chem.* **1996**, *68*, 849.
37. Pandey, G.; Dumbre, S. G.; Khan, M. I.; Shabab, M.; Puranik, V. G. *Tetrahedron Lett.* **2006**, *47*, 7923.

38. Vella, P.; Hussein, W. M.; Leung, E. W. W.; Clayton, D.; Ollis, D. L.; Mitic, N.; Schenk, G.; McGeary, R. P. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3282.
39. Faridoon; Hussein, W. M.; Vella, P.; Islam, N. U.; Ollis, D. L.; Schenk, G.; McGeary, R. P. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 380.
40. Ganellin, C. R.; Fkyerat, A.; Bang-Andersen, B.; Athmani, S.; Tertiuk, W.; Garbarg, M.; Ligneau, X.; Schwartz, J.-C. *J. Med. Chem.* **1996**, *39*, 3806.
41. Thomsen, R.; Christensen, M. H. *J. Med. Chem.* **2006**, *49*, 3315.
42. Guddat, L. W.; McAlpine, A. S.; Hume, D.; Hamilton, S.; de Jersey, J.; Martin, J. L. *Structure* **1999**, *7*, 757.
43. Crowder, M. W.; Spencer, J.; Vila, A. J. *Acc. Chem. Res.* **2006**, *39*, 721.