Med Chem Res 13:8/9 (2004) 565–577
© Birkhäuser Boston 2004

MEDICINAL CHEMISTRY RESEARCH

PHENYLPYRUVIC ACID DERIVATIVES AS ENZYME INHIBITORS: THERAPEUTIC POTENTIAL ON MACROPHAGE MIGRATION INHIBITORY FACTOR

Alain J.M. Carpy[1*], Petrus P. Haasbroek[2], Douglas W. Oliver[3]

[1] LPTC, UMR 5472 CNRS, University of Bordeaux 1, 351 Cours de la Libération F-33405 TALENCE CEDEX, France
[2] Dept of Pharmacy and Pharmacology, University of the Witwatersrand, 7 York Road, PARKTOWN 2193, Republic of South Africa
[3] Pharmacology, Faculty of Health Science, Potchefstroom University, POTCHEFSTROOM 2520, Republic of South Africa

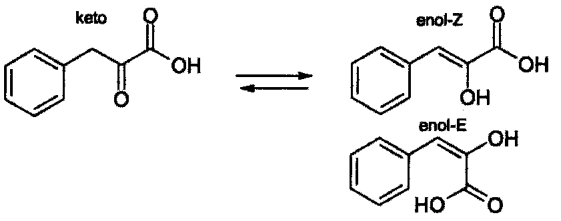
Abstract. Phenylpyruvic acid derivatives are obtained by hydrolysing aromatic Z/E azlactones. Keto tautomers interact with enzyme systems such as phenylalanine dehydrogenase or carboxypeptidase A whereas enol tautomers are potential inhibitors on the phenylpyruvate tautomerase activity catalysed by Macrophage Migration Inhibitory Factor (MIF). MIF being a key molecule in immune and inflammatory processes several structures with reasonable interaction with MIF and protocol for specific synthesis are presented.

Introduction

The incorporation of unnatural amino acids into peptides generates new pharmaceutical opportunities. Two synthetic approaches can be used: the azlactone route and the acetamidomalonic ester route. The azlactone route, reported as early as 1968[1], seems the most interesting one as it is cheap, easy and fast. It involves the hydrolysis of the azlactone to the corresponding phenylpyruvic acid intermediate which is converted to the phenylalanine. The

Corresponding author: email: a.carpy@lptc.u-bordeaux1.fr

literature has revealed a general lack of spectral data, in particular for the phenylpyruvic acid intermediates that have been reported. Furthermore, although the phenylpyruvic acids exhibit keto/enol tautomerism, has this phenomenon in the past been largely ignored by chemists in the planning of further synthetic reactions. For these reasons, we decided to re-investigate the azlactone route[2]. Aromatic azlactones are synthesised by condensing different ring-substituted benzaldehydes with an acylglycine (N-acetylglycine or hippuric acid) in the presence of acetic anhydride and anhydrous sodium acetate. These azlactones are then converted to the corresponding phenylpyruvic acids by hydrolysis under acidic or alkaline conditions yielding predominantly enol tautomers i.e. 2-hydroxy-3-phenylpropenoic acids and enol ester derivatives[3,4]. A direct route for the synthesis of novel acetates i.e. 2-acetoxy-3-phenylpropenoic acids has also been developed[5]. The conversion of phenylpyruvic acids to the phenylalanines proceeds via the keto tautomers. In addition, the azlactones can exist as Z/E isomers with a preference for the Z configuration[6]. The formation of the enol tautomers and their derivatives proceed via retention of configuration of the Z-geometry of the parent azlactones[3-5]. The E-isomers of the azlactones are more difficult to synthesise and require specific methods[7] (Scheme 1).



Scheme 1 : Phenylpyruvic acid as keto or Z/E-enol forms

Phenylpyruvic acid derivatives interact with several enzyme systems such as phenylalanine dehydrogenase (which catalyzes the oxidative deamination of L-Phe to form phenylpyruvic acid and ammonia with the concomitant reduction of NAD^+) or carboxypeptidase A (that hydrolyzes the carboxyl-terminal peptide bond in polypeptide chains). In the active sites of these two enzymes the phenylpyruvic acid derivatives exist as keto tautomers[8]. Interestingly these compounds are also potential inhibitors on the phenylpyruvate tautomerase activity

catalysed by the Macrophage Migration Inhibitory Factor (MIF). In the active site of MIF they exist as enol tautomers[9-10]. MIF has emerged as a potent pro-inflammatory cytokine and it has been implicated in a number of immune and inflammatory processes such as sepsis, acute respiratory distress syndrome, nephropathy and hypersensitivity reactions[11-12]. As phenylpyruvic acid derivatives exhibit specific stereochemical and tautomeric requirements in their interaction with the enzymes, we present drug design strategies taking these requirements into consideration. As MIF presents attractive opportunities for drug design and development with potential therapeutic applications[13-17], structures based on a theoretical study[18] with reasonable interaction with MIF and protocol for specific synthesis are also presented.

Chemistry

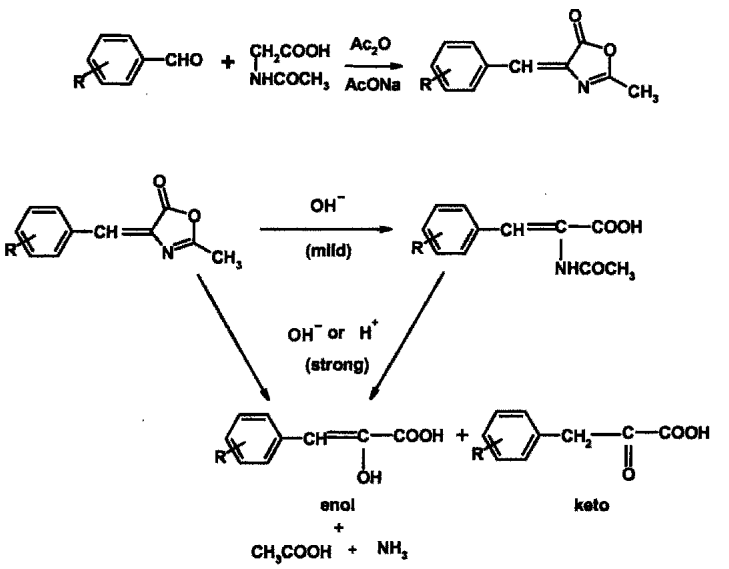
The Azlactone Route

Aromatic azlactones are synthesised from different substituted benzaldehydes (vanillin, isovanillin, veratraldehyde and their ring-halogenated derivatives, *o*- and *p*-chloro, fluoro or nitrobenzaldehyde etc), N-acetylglycine, acetic anhydride and anhydrous sodium acetate. ^{1}H and ^{13}C NMR and IR spectra together with single-crystal X-Ray analysis on selected compounds helped to elucidate the planar Z-configuration of the current series of compounds. These azlactones are converted to the corresponding phenylpropenoic acids by hydrolysis using strong acidic (HCl + AcOH) or strong alkalinic (20% NaOH) conditions according to Scheme 2.

Although the acid hydrolysis is preferred, the azlactones of vanillin, isovanillin and veratraldehyde having methoxy and hydroxyl substituent groups are found to undergo decomposition during acid hydrolysis, whereas the basic hydrolysis proceeds without decomposition. The chlorine atoms of their chlorinated derivatives seem to protect the ring from decomposition, as the acid hydrolysis of these azlactones proceeds smoothly without any decomposition. ^{1}H NMR spectral data of the synthesised phenylpyruvic acids show that the hydrolysis of the azlactones favour the formation of the enol tautomers i.e. 2-hydroxy-3-phenylpropenoic acids. However, the type and position of the substituent group in the phenyl ring have a definite influence on the ratio of keto/enol tautomer formation. Significant amounts of keto tautomers have been observed in hydrolysis products of azlactones with

moderate (Cl) or strong (NO_2) electron-withdrawing groups in the *ortho*-position of the phenyl ring. The *para*-substituted isomers form only minute quantities of the keto tautomers indicating the major role of the inductive effect in the equilibrium formation of the keto/enol tautomeric mixture when the electron-withdrawing group is in the *ortho*-position. For instance, the hydrolysis of the azlactone of the *o*-nitrobenzaldehyde gives a mixture of enol and keto tautomers of *o*-nitrophenylpyruvic acid while the hydrolysis of the azlactone of the *p*-nitrobenzaldehyde yields primarily the enol tautomer. ^1H NMR spectra of *o*-nitrophenylpyruvic acid at different temperatures in DMSO show that temperature has also an influence on the keto/enol ratio in solution. Increasing the temperature of the mixture increases the percentage of the keto form. The keto/enol ratio is also influenced by solvents with non-polar promoting the formation of keto tautomers. ^1H NMR spectrum of *o*-nitrophenylpyruvic acid in a less polar solvent such as $CDCl_3$ reveals the presence of a mixture of keto and enol tautomers.

The 2-acetoxy-3-phenylpropenoic acid and 2-benzoxy-3-phenylpropenoic acid compounds are directly synthesised from the corresponding azlactones by boiling it under reflux in acetic acid (80-90%). 2-Acetoxy-3-(4-hydroxyphenyl)-propenoic acid and 2-acetoxy-3-(4-acetoxyphenyl)-propenoic acids are also obtained using the alternative basic hydrolysis method (NaOH 20% under reflux). The hydrolysis of Z-azlactones proceeds with retention of the Z-configuration of the enol tautomers of phenylpyruvic acid derivatives.



Scheme 2: The azlactone route

Refined Synthetic Methods

• Synthesis of Z-azlactones

A mixture of the substituted benzaldehyde (0.1 mol), hippuric acid or N-acetylglycine (0.1 mol), anhydrous sodium acetate (0.2 mol) and acetic anhydride (30 - 40 ml) is heated at 80 to 110°C for 2 hours. The mixture is then poured into 100 ml water/ethanol (2:1) mixture, cooled in ice, filtered and washed with the water/ethanol mixture. The crude yellow product is purified by recrystallization from a suitable solvent such as benzene, chloroform, ethanol, methanol or a benzene/n-hexane mixture.

• Synthesis of E-azlactones

A mixture of the substituted benzaldehyde (0.1 mol), hippuric acid (0.1 mol) and polyphosphoric acid (120 g) is heated at 80 - 90°C for 2.5 hours. The mixture is poured into water (500 ml), stirred well, cooled in ice and filtered. The product is washed several times with water to get rid of the acid. The crude yellow product is recrystallized using a suitable solvent such as benzene, carbon tetrachloride, n-hexane or a mixed solvent system.

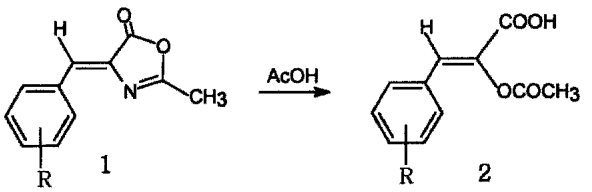
If a mixture of E and Z isomers is obtained, it is possible to isolate the E isomer by fractional crystallization.

• Synthesis of phenylpyruvic acids

The azlactone (1 g) is boiled under reflux in a mixture of glacial acetic acid (3 ml) and concentrated hydrochloric acid (7 ml) for 6 hours. The mixture is poured into water (10 ml) and cooled in a refrigerator overnight. Alternatively, the azlactone (1 g) is boiled under reflux in a sodium hydroxide solution (10 ml, 20%). After 2 hours, the mixture is cooled, acidified with hydrochloric acid (6 M) to a pH of 1.5 and cooled in a refrigerator overnight. The crystals that formed are filtered off and washed with water. Solvents such as benzene, ethanol, methanol, benzene/n-hexane or benzene/methanol mixtures can be used for recystallization. The phenylpyruvic acids (enol forms) obtained from both the Z and E isomers of the azlactones, have the same Z configuration.

• Synthesis of enol esters

A number of different ring substituted enol esters 2 were synthesized via their parent 2-methyl azlactones 1 (Z isomers). These syntheses were achieved by boiling the azlactones under reflux in strong acetic acid (90 – 96%), producing the enol acetates as shown in Scheme 3:

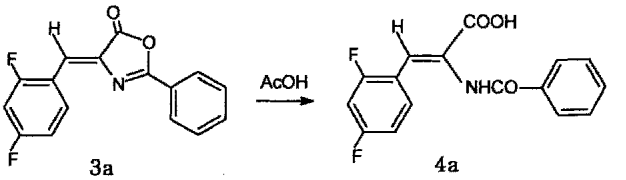


Scheme 3 : Acidic hydrolysis of a 2-methyl azlactone

As hydrolysis of the azlactones under strong acidic (conc. HCl / glacial acetic acid) or alkalinic (20% NaOH) conditions forms predominantly the enol form of the phenylpyruvic acid, we at first assumed that the mechanism of the ester formation proceeds via the enol form and therefore the acetate group of the ester originates from the acetic acid.

However our current study on the hydrolysis of 2-phenyl azlactones has proven this assumption wrong. Treatment of the 2-phenyl azlactone of 2,4-difluorobenzaldehyde 3a (Z

isomer) or 3b (E isomer) with acetic acid (95%) under the same conditions as with the 2-methyl azlactones, produced only the benzoyl amide 4a or 4b (Scheme 4). This is unique in the sense that previously the enol esters were obtained under these reaction conditions for non-aromatic (methyl) azlactones such as **2**. The aromaticity and the profound conjugated delocalised system between the phenyl and the azlactone ring of **3a** may be the energetically driving force to yield the amide instead of the enol ester (as was the case for non-aromatic (methyl) azlactones.



3a 4a

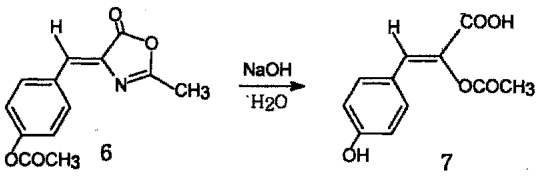
Scheme 4 : Acidic hydrolysis of a 2-phenyl azlactone

Furthermore, when the 2-methyl azlactone of 2,4-difluorobenzaldehyde **1** (R = 2,4-difluoro) is treated with propionic acid instead of acetic acid, the same product **2** (enol ester) was obtained (same melting point, same R_f values). These reactions clearly suggest that the ester group in the final product **2** originates from the azlactone structure and not the carboxylic acid reaction medium. It therefore appears that the mechanism(s) for these reactions involve(s) an intramolecular rearrangement reaction to form the enol esters. The reaction conditions seem to be more critical for the formation of the benzoyl esters than for the acetyl esters. It is important to note that when azlactones undergo mild hydrolysis, an amide product is formed and upon further strong hydrolysis, the amide is converted to the phenylpyruvic acid and ammonia.

Additionally in the case of the hydrolysis (95% acetic acid) of the E isomer of the azlactone **3b**, all the unreacted azlactone had the Z configuration indicating a conversion of configuration. Due to this transformation, a mixture of E and Z isomers of the benzoyl ester was formed which could be separated by fractional crystallization or column chromatography. The E isomer of the azlactone seems to hydrolyse more readily than the Z isomer, since the unreacted azlactone consisted of the Z isomer only. Dilution of the acetic acid seems to be

less favourable for this transformation as more of the E isomer of the ester appears to form with a 70% acetic acid mixture.

Recently, we reported on the formation of an enol acetate **7** that was obtained from the hydrolysis of the 2-methyl azlactone of p-hydroxybenzaldehyde **6** in 20% NaOH solution[7] (when a 2-methyl azlactone is synthesized from a *p*-hydroxybenzaldehyde, the hydroxy group is converted into an acetoxy group (Scheme 5)). This was not expected since under these strong alkaline hydrolysis conditions, the pyruvic acid (enol form) is normally formed.



Scheme 5 : Alkalinic hydrolysis of a 2-methyl azlactone

Similar results (enol acetate formation) were also obtained when the 2-methyl azlactone of 3-methoxy-4-hydroxybenzaldehyde was hydrolysed under the same strong alkaline conditions. These reactions suggest that groups such as OH (or OCOCH3) appear to be an important factor to yield enolate compounds. The mechanism of these two reactions appears to be similar to that of the acetic acid hydrolysis to form the enol esters.

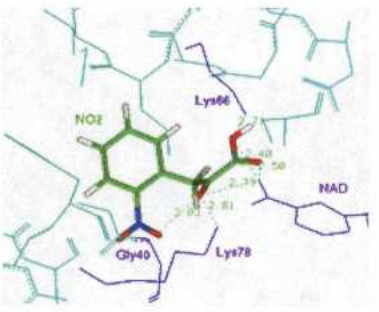
Molecular Modeling

Phenylpyruvic acid derivatives interact with several enzyme systems as described below. Phenylalanine dehydrogenase is a diagnostic enzyme which is used in the monitoring of phenylketonuria (PKU). It catalyzes the oxidative deamination of L-Phe to form phenylpyruvic acid and ammonia with the concomitant reduction of NAD followed by colorimetry. High-resolution X-ray analyses of an inhibitory ternary complex, namely the enzyme + NAD^+ + phenylpyruvate, revealed key features in the oxidative deamination mechanism[8]. In the active-site of the enzyme, the phenylpyruvate exists as the keto tautomer. Analysing the full characteristics of the active-site with different molecular modelling programs such as InsightII[19], LigPlot[20] or SURFNET[21] we demonstrated that it can

accommodate the *o*-nitrophenylpyruvate which exists solely as a ketone (Fig. 1). In addition, we also demonstrated that the active site is able to accommodate the ester acetate.

Carboxypeptidase A (CPA) is a pancreatic exopeptidase hydrolyzing the peptide bond adjacent to the C-terminal end of a polypeptide chain. Following preliminary studies performed on potential inhibitors of the bovine CPA[1], the structure of which having recently been refined[22], we demonstrated that the human CPA[23] might be complexed by phenylpyruvates as keto forms (Fig. 2).

Phenylpyruvate derivatives can also be potential inhibitors of the phenylpyruvate tautomerase activity catalysed by the Macrophage Migration Inhibitory Factor (MIF). MIF has first been identified as a pro-inflammatory cytokine released under a variety of circumstances from T-cells and macrophages and as a key molecule in inflammation.

1 

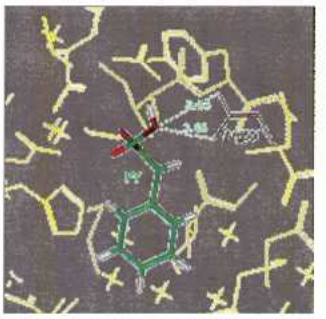
2 

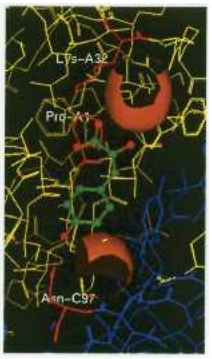
Fig. 1 The keto *o*-nitrophenylpyruvate docked into the active site of the Phe-dehydrogenase

Fig. 2 The keto form of the phenylpyruvate docked into the active site of CPA

In recent years it has also been shown that MIF assumes an important role as a regulator of innate and acquired immunity. MIF been identified as a new therapeutic target for inflammatory and autoimmune diseases, MIF-directed therapies might offer new treatment opportunities for human diseases. Contrary to the complexes with the two previous enzymes, phenylpyruvate derivatives exist in the active site of MIF as enol tautomers[9-10]. Not only do the keto/enol tautomers differ with respect to the position of the hydrogen atom in

thesetautomers, but they also have different conformations, almost planar for the enol forms and definitively non-planar for the keto forms. In addition, the (E)-2-fluoro-p-hydroxycinnamate was found to be a more potent inhibitor than the (Z)[10]. The unique structural features of these compounds, including phenylpyruvate tautomerase activity and D-dopachrome tautomerase activity, prompted us to design new potential therapeutic MIF inhibitors. A detail of the SURFNET plot showing the protein-inhibitor complex (MIF-(E)-*p*-hydroxyphenylpyruvic acid) and the gap regions between the two is given Fig. 3-a. The gap regions (brown) indicate that the possibilities for modifying the phenylpyruvic acid structure may occur at one *meta* position (and to a lesser extent at one *ortho* position) and also at the acid functionality (place for an ester function). Several aromatic ring substitution modelling studies have been conducted. The results clearly indicate that the narrow active site of the MIF enzyme can accommodate sterically small substitution patterns. These include fluoro substitution at the *meta* and *ortho* positions. Substitution with chloro groups or sterically similar methyl groups indeed proved to be sterically unfavourable. Molecular overlap was also found for enol-acetate esters, indicating that ester forms could not also interact with the enzyme but could be used as useful pro-drugs for drug delivery purposes. Azlactones (oxazolones) derivatives that were modelled in the tautomerase active site of MIF show significant molecular overlap between the aromatic moieties and other functionalities of *p*-hydroxyphenylpyruvate and 2-fluoro-*p*-hydroxycinnamate. The oxazolone moiety indeed proved to be a useful isoster replacement for the acid and enol hydroxy functionalities. Similar pharmacophore interactions were found for these azlactone compounds. It is suggested that identical aromatic ring substitution patterns will be accommodated for the azlactones as were observed for the acid derivatives (Fig. 3-b).

3-a



3-b

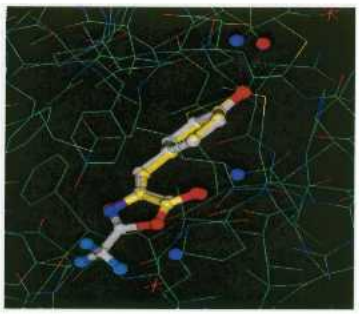


Fig. 3-a A SURFNET plot of the complex MIF-(E)-*p*-hydroxyphenylpyruvic acid.

Fig. 3-b A 2-methyl azlactone docked into the active site of MIF

Conclusion

Our investigation successfully explored the tautomeric and stereochemical aspects of new compounds of the 2-phenylpyruvic acid type synthesized via the azlactone route. Spectroscopic methods such as MNR, IR and X-ray were used to establish the structures of these compounds. When azlactones undergo mild hydrolysis generally amides are formed while strong hydrolysis yields phenylpyruvic acids. However, under acetic acidic or alkalinic conditions, enol esters are formed except in the case where a phenyl moiety is present that promotes a conjugated delocalization system to the azlactone ring, which appears to contribute to the formation of the amide and not the expected enol ester. Using strong carboxylic acid conditions, we have a clear demonstration that the ester group comes from the azlactone and not from the acid reaction medium. A yet unknown intramolecular rearrangement seems to give the most probable answer to explain the hydrolysis to form these esters. Finally, using a strong mineral acidic medium directly yields phenylpyruvic acids.

The interactions of these compounds with different enzymes such as the phenylalanine dehydrogenase, the carboxypeptidase A and MIF have also been explored. In particular molecular modelling studies suggested promising interaction with MIF for some of them. This has to be confirmed by pharmacological tests and single crystal X-ray crystallography on the corresponding complexes.

Acknowledgements

The financial support from the French Government and National Research Foundation of South Africa is gratefully acknowledged.

References

1. Crooij, P. South African Patent 68,3083, 1968.
2. Haasbroek, P.P. Ph.D. thesis, University of Potchefstroom, SOUTH AFRICA, 1997.
3. Oliver, D.W.; Haasbroek, P.P.; Léger, J.M.; Carpy, A.J.M. *J. Chem. Cryst.* **1994**, *24*, 665-668.
4. Haasbroek, P.P.; Oliver, D.W.; Carpy, A.J.M. *J. Chem. Cryst.* **1998**, *28*, 193-196.
5. Haasbroek, P.P.; Oliver, D.W.; Carpy, A.J.M. *J. Chem. Cryst.* **1998**, *28*, 139-143.
6. Haasbroek, P.P.; Oliver, D.W.; Carpy, A.J.M. *J. Chem. Cryst.* **1998**, *28*, 811-814.
7. Haasbroek, P.P.; Oliver, D.W.; Carpy, A.J.M. *J. Mol. Struct.* **2003**, *648*, 61-67.
8. Vanhooke, J.L.; Thoden, J.B.; Brunhuber, N.M.; Blanchard, J.S.; Holden, H.M. *Biochemistry* **1999**, *38*, 2326-2334.
9. Lubetsky, J.B.; Swope, M.; Dealwis, C.; Blake, P.; Lolis, E. *Biochemistry* **1999**, *38*, 7346-7354.
10. Taylor, A.B.; Johnson, W.H. Jr.; Czerwinski, R.M.; Li, H.-S.; Hackert, M.L.; Whitman, C.P. *Biochemistry* **1999**, *38*, 7444-7452.
11. Baugh, J.A.; Bucala, R. *Crit. Care Med.* **2002**, *30*, S27-35.
12. Lolis, E.; Bucala, R. *Expert Opin. Ther. Targets* **2003**, *7(2)*, 153-164.
13. Orita, M.; Yamamoto, O.M.; Katayama, N.; Fujita, S. *Curr. Pharm. Des.* **2002**, *8*, 1297-1317. (A review with 133 references).
14. Senter, P.D.; Al-Abed, Y.; Metz, C.N.; Benigni, F.; Mitchell, R.A.; Chesney, J.; Han, J.; Gartner, C.G.; Nelson, S.D.; Todaro, G.J.; Bucala, R. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 144-149.
15. Lubetsky, J.B.; Dios, A.; Han, J.; Aljabari, B.; Ruzsicska, B.; Mitchell, R.; Lolis, E.; Al-Abed, Y. *J. Biol. Chem.* **2002**, *277*, 24976-24982.
16. Dios, A.; Mitchell, R. A.; Aljabari, B.; Lubetsky, J.; O'Connor, K.; Liao, H.; Senter, P.D.; Manogue, K.R.; Lolis, E.; Metz, C.N.; Bucala, R.; Callaway, D.J.E.; Al-Abed, Y. *J. Med. Chem.* **2002**, *45*, 2410-2416.
17. Carpy, A.J.M.; Haasbroek, P.P.; Oliver, D.W. *J. Chin. Chem.* **2003**, *21*, 1256-1262.

18. Carpy, A.J.M.; Haasbroek, P.P.; Ouhabi, J.; Oliver, D.W. *J. Mol. Struct.* **2000**, *520*, 191-198.
19. InsightII, Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752, USA
20. Wallace, A.C., Laskowski, R.A. and Thornton, J.M. LIGPLOT, *Prot. Eng.*, **1995**, *8*, 127-134.
21. Laskowski, R.A. SURFNET, *J. Mol. Graph.* **1995**, *13*, 323-330.
22. Kilshtain-Vardi, A.; Glick, M.; Greenblatt, H.M.; Goldblum, A. Shoham, G. *Acta Cryst.* **2003**, *D59*, 323-333.
23. Reverter, D.; Fernandez-Catalan, C.; Baumgartner, R.; Pfander, R.; Huber, R.; Bode, W.; Vendrell, J.; Holak, A.; Aviles, F. *Nat. Struct. Biol.* **2000**, *7*, 322-328.