Design of Agents Interacting with Immunoregulating Proteins: Potential Inhibitors of the Phenylpyruvate Tautomerase Activity Catalysed by Macrophage Migration Inhibitory Factor (MIF)

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The macrophage migration inhibitory factor has been implicated in a number of immune and inflammatory processes. MIF presents particular opportunities for drug design and development with potential therapeutic applications. Drug design strategies taking into consideration of specific stereochemical and tautomeric requirements in the interaction of MIF with substrates and inhibitors allow several novel structures to be designed. Our investigations successfully explored the tautomeric and stereochemical aspects of new compounds of the 2-phenylpyruvic acid type, both experimentally, through synthesis and structural investigations and computationally, through molecular mechanics and quantum mechanics calculations.[1]

Keywords macrophage migration inhibitory factor, phenylpyruvate tautomerase activity, azlactone, phenylpyruvic acid derivative

Introduction

This study started in our group, a few years ago, with the investigation of the azlactone route as a suitable method for the synthesis of unnatural amino acids of the phenylalanine type via the phenylpyruvic acid intermediates. The idea was to incorporate unnatural amino acids into peptides, thereby generating new medical and pharmaceutical opportunities.[1] Even if the azlactone route was reported as early as 1968,[2] literature has revealed a general lack of spectral data for the azlactones and for the phenylpyruvic acid intermediates that have been reported. Furthermore, although the phenylpyruvic acids exhibit keto/enol tautomerism, this phenomenon has seemingly been largely ignored by researchers in the planning of further synthetic reactions. A number of selected aromatic azlactones were successfully synthesized from benzaldehyde and different ring substituted benzaldehydes, *N*-acetylglycine or hippuric acid, acetic anhydride and anhydrous sodium acetate. Then these azlactones were converted to the corresponding phenylpyruvic acids by hydrolysis, using strong acid (HCl + AcOH) or strong alkali (20% NaOH). In both cases, the hydrolysis yields predominantly the enol tautomers (2-hydroxy-3-phenylpropenoic acids) while the conversion of 2-phenylpyruvic acids to the phenylalanines proceeds via the keto tautomers.

The structural elucidation of the synthesised compounds was done by using ^{1}H NMR, ^{13}C NMR, IR spectra and single crystal X-ray analysis.[3-6] The keto/enol tautomerism and *Z*/*E* isomerism of the enol forms were studied with theoretical methods using quantum mechanics calculations.[7] Some promising results were obtained on preliminary biological evaluations performed on selected compounds, using various isolated organ preparations.[1] The most promising response was obtained for the 2-acetoxy-3-phenylpropenoic acid, for reduction of serotonin response on the aorta via a non-specific mode of action. Tests done on the guinea-pig ileum showed that enols exhibit weak antagonism of the action of histamine. A molecular modelling study indicated that the keto forms have potential as inhibitors of carboxypeptidase A.[1]

More recently we further developed new phenylpyruvic acid derivatives on the basis of potential inhibitors on the phenylpyruvate tautomerase activity catalysed by Macrophage Migration Inhibitory Factor (MIF). MIF has emerged as a potent pro-inflammatory cytokine released from T-cells and macrophages, and it has been implicated in a number of immune and inflammatory processes.[8] An unusual property uncovered for MIF is its ability to catalyse keto/enol tautomerisation reactions. MIF catalyses the enolisation of *D*-dopachrome to generate the 5,6-dihydroxyindole-2-carboxylic acid (Scheme 1)[9] (MIF exhibits similar tautomerase activity as human *D*-dopachrome tautomerase). Recently, phenylpyruvate and *p*-hydroxyphenylpyruvate have also been found to be MIF substrates, and it was further concluded that MIF and phenylpyruvate tautomerase are the same protein[10] even if it remains unclear whether there is a relationship between the phenylpyruvate tautomerase activity of MIF and its other biological activity.[11-14] However these findings prompted

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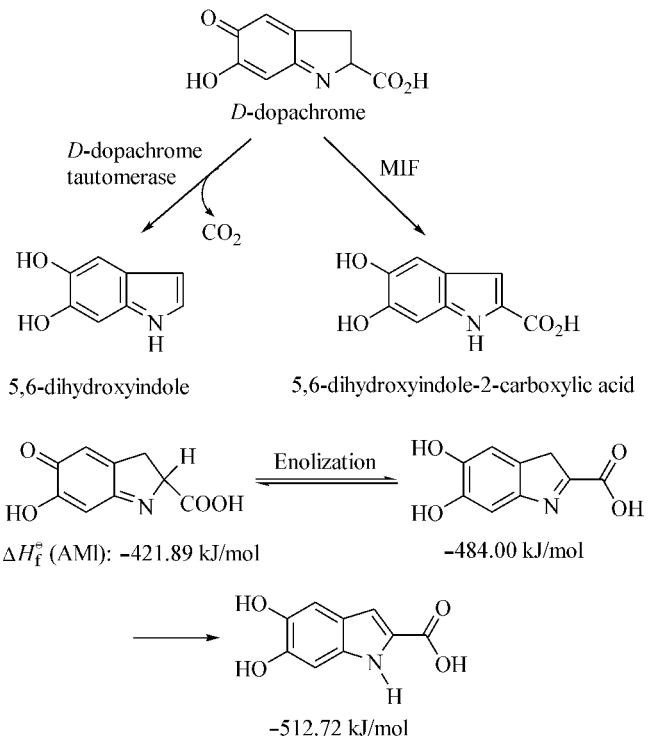
some studies on the design of tautomerase inhibitors.[14-19]

Scheme 1 Tautomerase activity of human MIF similar to human *D*-dopachrome tautomerase activity



The three-dimensional structure of MIF is unlike any known cytokine but shows similarities with three microbial enzymes: 4-oxalocrotonate tautomerase, 5-carboxymethyl-2-hydroxymuconate isomerase and chorismate mutase. Three monomers associate to form a trimer. Each monomer possesses two antiparallel α-helices that pack against a β-sheet (Fig. 1).[20-22] Crystal structures of MIF complexed with *p*-hydroxyphenylpyruvate[23] and 2-fluoro-*p*-hydroxycinnamate[24] were determined and identified Pro-1, Lys-32 from one monomer, Tyr-95, and Asn-97 from the adjacent monomer, as active site residues whereas Met-2, Tyr-36, Ile-64, Val-106 and Phe-113 form an hydrophobic environment (Fig. 2). Crystal structures of MIF complexed with competitive inhibitors have shown that the substrates exist as enol forms (2-hydroxy-3-phenylpropenoic acids).[23,24] On the contrary the phenylpyruvic acid in the active site of another enzyme (phenylalanine dehydrogenase) exists as the keto form[25] (Fig. 3). Not only do the keto/enol tautomers differ in the point of attachment of a hydrogen atom but they also have different conformations, almost planar for the enol forms and non-planar for the keto forms. The enol tautomers can exist with two different configurations *Z*/*E*. It was found that the (*Z*)-*p*-hydroxycinnamate is a more potent inhibitor than its isomeric counterpart *E* and that the (*E*)-2-fluoro-*p*-hydroxycinnamate is a more potent inhibitor than its isomeric counterpart *Z*. Its seems somewhat confusing but in fact the *Z*

Fig. 1 Ribbon representation of MIF as a trimer, showing mainly antiparallel β-sheets with segregated α and β regions.

Fig. 2 Three active sites of MIF from Ref. 24. The residues involved in each site are Pro red, Lys blue (from one monomer), Tyr green, Asn purple (adjacent monomer).

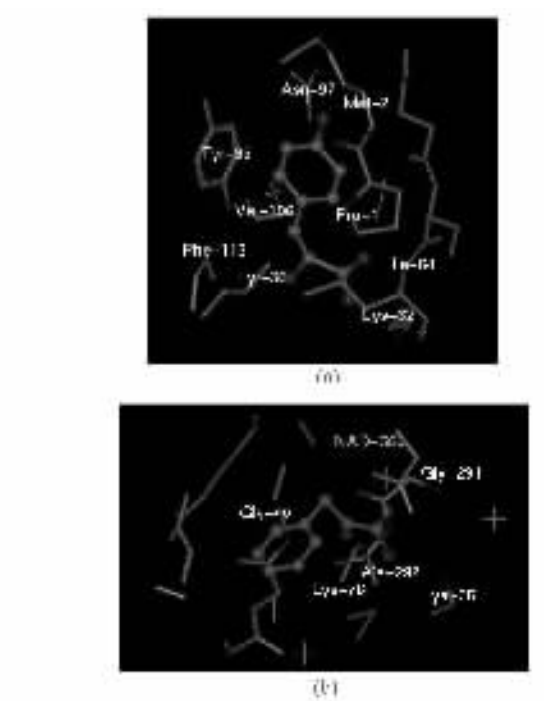
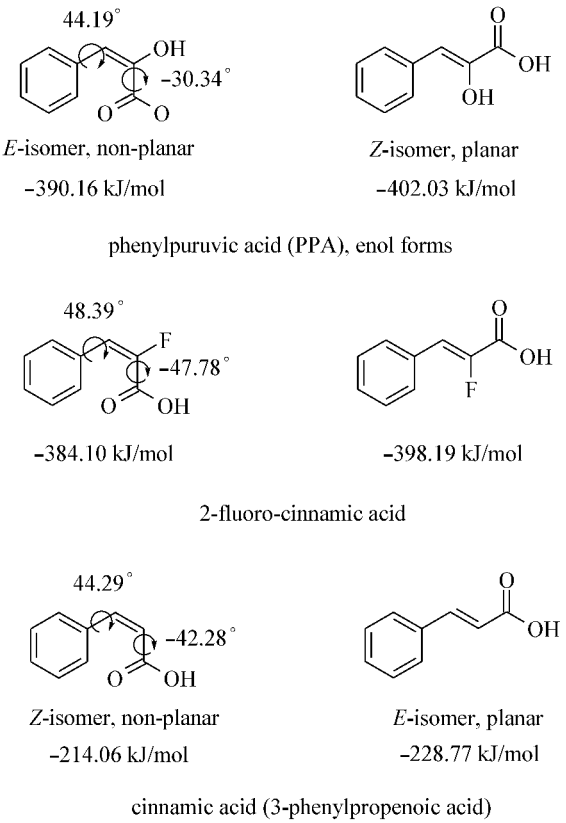


Fig. 3 MIF vs. phenylalanine dehydrogenase. In the active site of MIF, competitive inhibitors exist as enol forms (a). On the contrary phenylpyruvic acid derivatives in the active site of phenylalanine dehydrogenase, exist as keto forms (b).

configuration of the *p*-hydroxycinnamate is three dimensionally identical to the *E* configuration of the 2-fluoro-*p*-hydroxycinnamate (Scheme 2). The nomenclature notation preferences for substituents is responsible for this apparent discrepancy. Our contribution in this field is reported here.

Scheme 2 Similarities between the geometrical isomers of the enol forms of phenylpyruvic acid, and of cinnamic and 2-fluoro-cinnamic acids



Aim and objectives

Potent tautomerase inhibitors would be useful (i) to highlight the role of MIF activity and the link between its biological and enzymatic activities (ii) as potential drugs in the treatment of a variety of inflammatory and immune MIF-related diseases such as sepsis, acute lung injury, rheumatoid arthritis, glomerulonephritis, and tumors.

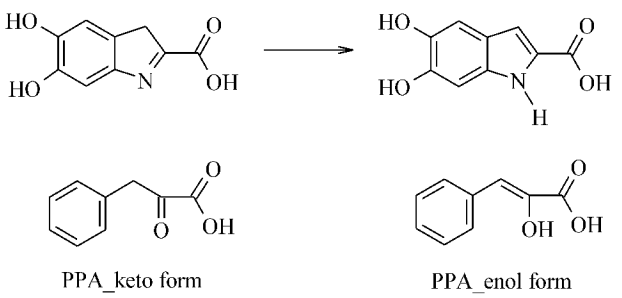
Using available structural knowledge on the MIF catalytic site, molecular modelling and docking techniques, we report herein the design and syntheses of new phenylpyruvic acid derivatives as potential inhibitors of the phenylpyruvate tautomerase activity and possible new drugs. The azlactone route was chosen as a suitable method for the synthesis of these compounds. Tautomerism and the influence of substituent groups and their positions in the phenyl ring on the keto/enol formation of phenylpyruvic acid derivatives had to be carefully examined. For that, we used the classical experimental tools such as NMR, IR spectra and X-ray crystal structure analysis but also quantum mechanics calculations. Taking advantage of our expertise in the preparation of enol tautomers, we report the methods of separation of the *Z*/*E* forms.

Molecular modelling studies

Quantum mechanics calculations

During the enolisation of *D*-dopachrome, the first intermediary structure formed is equivalent to a locked conformation of a phenylpyruvic acid as keto form, whereas the final 5,6-dihydroxyindole-2-carboxylic acid is equivalent to a locked conformation of a phenylpyruvic acid as (*Z*)-enol form [(*Z*)-2-hydroxy-3-phenylpropenoic acid] (Scheme 3), showing strong similarities between the *D*-dopachrome tautomerase activity of MIF and its phenylpyruvate tautomerase activit. The similitude between *D*-dopachrome and phenylpyruvate was also evidenced using semi-empirical quantum mechanics calculations and the AMPAC program.[26] The standard enthalpies of formation ΔH_f^0 were calculated for all possible forms of dopachrome (Scheme 4) showing that all enol forms are more stable than the two possible keto forms. The calculated enthalpies are in good agreement with experimental findings. The enolisation reaction of *D*-dopachrome catalysed by MIF leads to 5,6-dihydroxyindole-2-carboxylic acid whose structure corresponds to the (*Z*)-2-hydroxy-3-phenylpropenoic acid which is less stable by *ca*. 5.434 kJ/mol than the initial keto form.

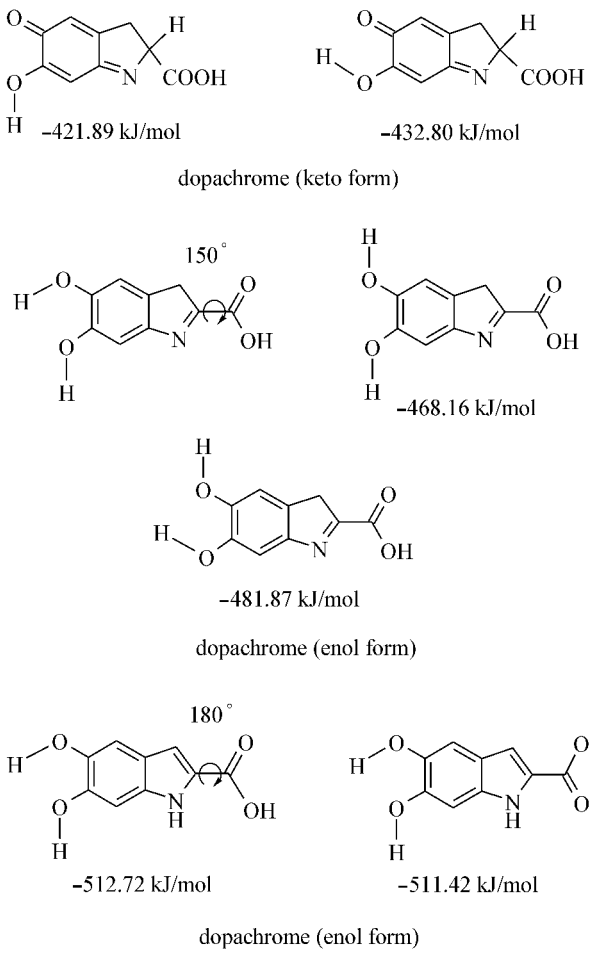
Scheme 3 Similarities between tautomeric forms of *D*-dopachrome and tautomeric forms of phenylpyruvate



Concentrating on enol forms, the similarities between low-energy conformations, configurations, and enthalpies of phenylpyruvic acid (2-hydroxy-3-phenylpropenoic acid), cinnamic acid, and 2-fluoro-cinnamic acid are shown in Scheme 2. The planar *Z*-isomers of 2-hydroxy-3-phenylpropenoic acid and 2-fluoro-3-phenylpropenoic acid (2-fluoro-cinnamic acid) are more stable than their non-planar *E*-counterparts (the torsion angles at both sides of the double bond being 44.19° and −30.34° for (*E*)-2-hydroxy-3-phenylpropenoic acid and 48.39° and −47.78° for (*E*)-2-fluoro-3-phenylpropenoic acid. Similarly, the planar (*E*)-isomer of 3-phenylpropenoic acid is more stable than its non-planar *Z*-counterpart (the torsion angles

at both sides of the double bond being 44.29° and −42.28°).

Scheme 4 Enthalpies standard of formation ΔH_f^0 for all possible forms of dopachrome



Docking studies

The pharmacophoric groups that are important, in the interaction of these compounds with MIF, are the *p*-hydroxyl group which forms a hydrogen bond with Asn-97 and the acid functionality interacting with Lys-32 and Ile-64 via two other hydrogen bonds. It was recently shown that the *p*-hydroxyl group is required to maintain the inhibitory activity and that replacement by a halide or a methyl group resulted in complete loss of inhibitory effect.[19] Other important residues of the active site include a hydrophobic pocket Met-2, His-62, Met-101, Val-106 and Phe-113. The first approach that we used was to dock ring-substituted phenylpyruvic acid derivatives as enol forms into the tautomerase active site. Gaps regions between *p*-hydroxyphenylpyruvate and tautomerase were calculated by using SURFNET[27] and represented by using the InsightII graphics facilities[28] (Fig. 4). From this result it seems 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. Several aromatic ring substitution modelling studies have been conducted. These results clearly indicated that the narrow active site of the enzyme can accommodate sterically small substitution patterns. These include fluoro substitution at the *meta* and *ortho* positions. Fig. 5

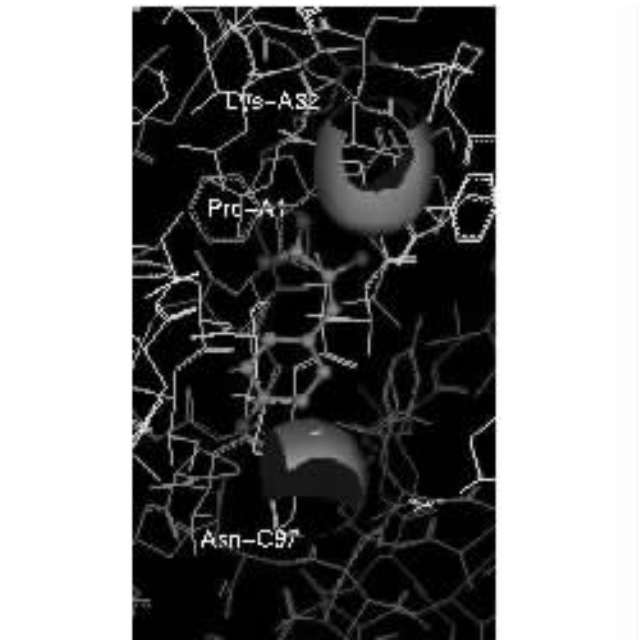


Fig. 4 Gaps (brown) showing possible modification of *p*-hydroxyphenylpyruvic acid. Calculations and representation using SURFnet and its graphic interface with InsightII.

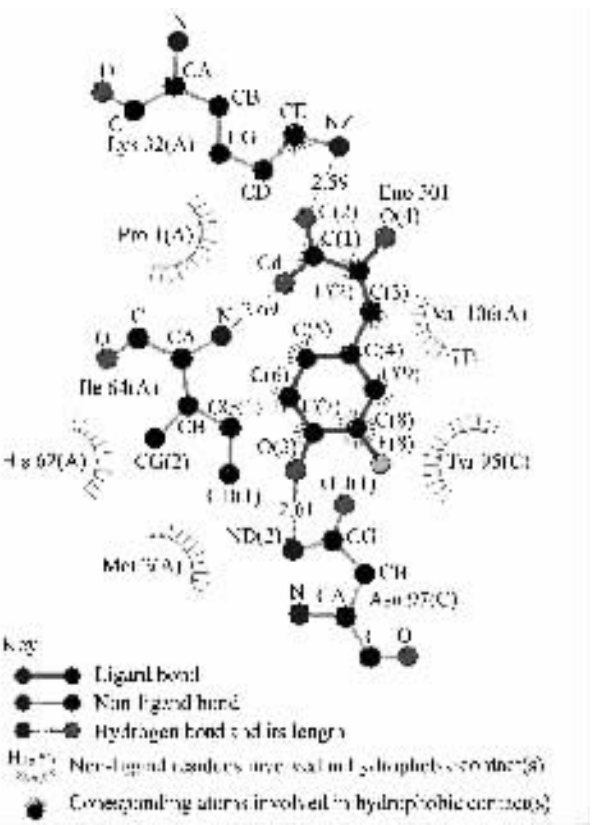


Fig. 5 Results of the docking of 3-fluoro-4-hydroxy-phenylpyruvic acid using LIGPLOT.

shows a schematic 2*D*-representation of possible interactions between 3-fluoro-*p*-hydroxyphenylpyruvate and the active site of MIF, using LIGPLOT.[29] Substitution with chloro groups or sterically similar groups (methyl) 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 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 azlactones as were observed for the acid derivatives.

Tautomerism and geometrical isomerism

Experimental studies

Tautomerism

Tautomerism plays a major role in the phenylpyruvic acid derivatives formation. In the formation of pyruvic acids from the corresponding azlactones, the enol form is formed first, which can then tautomerise to the more stable keto form, a phenomenon which is well known for enols in general. In the literature, when reference is made to a phenylpyruvic acid, it is generally assumed to be the keto form. In the present study, it was found that the synthesis of the phenylpyruvic acids from the azlactone route, favoured the formation of the enol in the unsubstituted as well as in different substituted phenylpyruvic acids. The type and position of the substituent group in the phenyl ring have a definite influence on the ratio of enol/keto tautomer formation. Significant amounts of the keto tautomers could only be 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 formed only minute quantities of the keto tautomers, indicating the major role of the inductive effect in the equilibrium formation of the enol/keto tautomeric mixture when the electron-withdrawing group is in the *ortho*-position. For instance, the hydrolysis of the azlactone of *o*-nitrobenzaldehyde gave a mixture of enol and keto tautomers of *o*-nitrophenylpyruvic acid. However, *p*-benzaldehyde yielded primarily the enol tautomer of nitrophenylpyruvic acid, with only trace amounts of the keto tautomer being detected in its ^{1}H NMR spectrum. A singlet at δ 6.70 was observed for the benzylic proton of the enol form of *o*-nitrophenylpyruvic acid, and a singlet at δ 4.65 was observed for the benzylic protons of the keto form of *o*-nitrophenylpyruvic acid. The presence of the keto form was supported by the ^{13}C NMR spectrum of *o*-nitrophenylpyruvic acid which showed the extra signals of the two forms, the signals at δ 43.7 (benzylic carbon) and δ 192.2 (α-carbonyl carbon) clearly showed those of the keto forms. Calculations based on the peak intensities of the ^{1}H NMR spectrum of *o*-nitrophenylpyruvic acid showed that approximately 59% of the enol form and 41% of the keto form are present in the tautomeric mixture in DMSO at room temperature. The ^{1}H NMR spectra of *o*-nitrophenylpyruvic acid recorded at different temperatures, in DMSO, showed that temperature has an influence on the enol/keto ratio in solution. Increasing the temperature of this mixture increased the percentage of the keto form. At 40 ℃ the ratio was 53% enol form and 47% keto form, while at 60 ℃ the enol form decreased further to 46% and the keto form increased to 54%. The enol/keto ratio is also influenced by solvents. Non-polar solvents seem to promote formation of the keto isomer. The ^{1}H NMR spectrum of *o*-nitrophenylpyruvic acid in the less polar solvent $CDCl_3$, revealed the presence of 37% of the enol tautomer and 63% of the keto tautomer at room temperature. At 42 ℃ the ratio changed to 32% enol form and 68% keto form. Also noteworthy is the downfield shift of the signal of the benzylic proton in $CDCl_3$ (δ 7.26), compared to the value of δ 6.7 for the same proton in DMSO.

Geometric isomerism

Another interesting feature is the formation of specific geometrical isomers for the azlactones, enol tautomers of phenylpyruvic acids and enolic acetate esters. Due to the presence of an olefinic bond in the azlactones, the two geometrical isomers, *Z* and *E*, are possible. It is now generally accepted that the thermodynamically more stable *Z*-isomers (carbonyl group of azlactone ring *trans* to phenyl group of the benzilidene moiety) are obtained when aromatic aldehydes are condensed with *N*-acetylglycine or hippuric acid in the presence of acetic anhydride and sodium acetate.[30-35] NMR spectra and X-ray crystallography studies showed that the solvolysis of azlactones proceeds with retention of configuration.

Theoretical calculations

The relative stabilities of the different tautomers and geometrical isomers were confirmed by quantum mechanics calculations *in vacuo*.

The ratio of two tautomers is described by the equilibrium constant K_T, which is classically calculated from their free energy difference ΔG:

$$K_T = \exp(-\Delta G/RT) \quad (1)$$

ΔG is expressed as a function of enthalpy difference ΔH and entropy difference ΔS:

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

The enthalpy difference ΔH at 298 K is given by:

$$\Delta H^{298} = \Delta E_T + \Delta E_v^0 + \Delta(\Delta E_v^{298}) + \Delta E_r^{298} + \Delta E_t^{298} + \Delta(PV) \quad (3)$$

E_T is the total energy, E_v^0 is the zero-point energy (ZPE), ΔE_v^{298} is the change in vibrational energy when T increases from 0 to 298.15 K, E_r^{298} and E_t^{298} are the rotational and translational energies and PV is the work term.

In tautomeric equilibria, the last 3 terms of Eq. (3) are all equal to zero.

For instance, *ab initio* calculations showed a large preference for the enol tautomers relative to the keto tautomers in the case of phenylpyruvic acid and of *o*-chlorophenylpyruvic acid (K_T = 624.8 and 326.8 respectively). Conversely, the *o*-nitrophenylpyruvic acid exists preferentially as a ketone ($K_T = 1.73 \cdot 10^{-3}$).[7]

The differences in stability between the geometrical isomers were simply calculated by using semi-empirical methods and AMPAC. For instance ΔH_f^0 for the enol form of phenylpyruvic acid is in favour of the planar *Z*-configuration vs. the non-planar *E*-configuration by *ca*. 12.54 kJ/mol.

Implications of tautomerism and geometrical isomerism with respect to the biological activity and specificity

The formation from the azlactones, of the predominantly enol tautomers of phenylpyruvic acids, can have important implications if used for further reactions. But tautomerism and geometrical isomerism of these compounds should also be considered for the rational design of enzyme inhibitors or ligand-receptor interactions studies. As such simple molecules act on different targets as enol forms (MIF) or keto forms (phenylalanine dehydrogenase), complexes with other enzymes such as *D*-dopachrome tautomerase[36] should be carefully investigated.

Refined synthetic methods

The synthetic methods of preparing specifically the *Z*- and *E*-isomers of the azlactones and their phenylpyruvic acid and enol acetate derivatives are illustrated.

Synthesis of azlactones (Z-isomer)

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) was heated at 80 ℃ to 110 ℃ for 2 h. The mixture was then poured into 100 mL of *V* (water)/*V* (ethanol) (2:1) mixture, cooled in ice, filtered and washed with the water/ethanol mixture. The crude yellow product was purified by recrystallization from a suitable solvent such as benzene, chloroform, ethanol, methanol or a benzene/*n*-hexane mixture.

Synthesis of azlactones (E-isomer)

A mixture of the substituted benzaldehyde (0.1 mol), hippuric acid (0.1 mol) and polyphosphoric acid (120 g) is heated at 80—90 ℃ for 2.5 h. 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 by 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 the phenylpyruvic acids

Azlactone (1 g) was boiled under reflux in a mixture of glacial acetic acid (3 mL) and concentrated hydrochloric acid (7 mL) for 6 h. The mixture was poured into water (10 mL) and cooled in a refrigerator overnight. Alternatively, azlactone (1 g) was boiled under reflux in a sodium hydroxide solution (10 mL, 20%). After 2 h, the mixture was cooled, acidified with hydrochloric acid (6 mol/L) to a pH of 1.5 and cooled in a refrigerator overnight. The crystals formed were 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 form) obtained from both the *Z* and *E* isomers of azlactones, have the same *Z* configuration.

Synthesis of the enol acetate esters

2-Methyl azlactone (obtained by using *N*-acetylglycine) (1 g) was boiled under reflux in acetic acid (10 mL, 90%) for 4 h. Water (10 mL) was added and the mixture was cooled in ice, filtered and washed with water. The compound can be recrystallized from solvents such as ethanol, methanol, benzene or benzene/methanol mixture.

Conclusions and perspectives

The macrophage migration inhibitory factor presents unique opportunity for drug design and development with potential therapeutic applications. The multiple mechanism involved in the tautomeric catalytic activities of MIF, however, is still being investigated and in the process of being elucidated. This unique trimeric protein exhibits specific stereochemical and tautomeric requirements in its interaction with substrates and inhibitors. This implies that drug design strategies must take these requirements into serious consideration to be successful in developing new structures. Several novel structures have subsequently been designed with reasonable interaction with MIF. However, there is considerable scope for medicinal synthetic chemists and drug design modellers to explore the protein.

Our investigations successfully explored the tautomeric and stereochemical aspects of new compounds of the 2-phenylpyruvic acid type, both experimentally, through synthesis and structural investigations, and computationally, through molecular mechanics and quantum mechanics calculations. Additionally, our molecular modelling of these structures suggests promising interaction with MIF.

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