A Model of the Interaction of Substrates and Inhibitors with Xanthine Oxidase

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Abstract: A model of the interaction of substrates and inhibitors with xanthine oxidase (XO) based on similarity concepts and molecular modeling is introduced and discussed, and previous literature is reexamined in the light of recent insights into the mechanism and structure of XO. Use is made of quantum-chemical calculations with the inclusion of solvent effects, molecular superimposition with least-squares fitting algorithms, and molecular electrostatic potentials. First, the relative stabilities of the tautomeric forms of the physiological substrates, xanthine and hypoxanthine, are calculated both *in vacuo* and in water in order to select the most abundant form(s) at physiological pH: the two substrates prove to be stable in their lactam forms, with a dominance of the N_7-H tautomer for xanthine and of N_9-H for hypoxanthine. The structures of xanthine and hypoxanthine are then superimposed, and their relative orientation with respect to the molybdenum center of XO is suggested. The criteria used for superimposition reflect the importance of functional groups of xanthine and hypoxanthine, as inferred from experimental work. In particular, the carbonyl oxygen common to the two substrates is given special consideration on account of its determinant role. The results show that the most important functional groups of the two substrates can be successfully superimposed by means of a rotation that exchanges the five-membered with the six-membered rings of xanthine and hypoxanthine with respect to molybdenum. The close similarity of the electrostatic potentials of the two superimposed molecules adds weight to the proposed orientation of the substrates in the binding site. The model of interaction is then tested and further developed using a series of previously-synthesized dimensional analogs of xanthine and hypoxanthine. The results confirm that the correct positioning of the carbonyl group is essential if a productive interaction with XO is to be achieved and allow us to map the dimensions of the active site starting from the superimposition of the physiological substrates. Two hypotheses regarding the amino acid residues interacting with the important carbonyl oxygen of the substrates are then put forward on the basis of spectroscopic and biochemical evidence: they are postulated to be one lysine or one protonated glutamic acid residue. In an attempt to unify the binding of substrates and inhibitors, the model is extended to the inhibitors of XO by superimposing the most interesting inhibitors developed by Robins on xanthine and hypoxanthine. This allows us to define the most suitable location of the phenyl rings of these inhibitors with respect to the superimposition of the substrates. Intriguingly, the superimpositions of the most active inhibitors are consistent with a unique location of their phenyl rings, even though they are in different positions on the purine ring. Finally, the flavone, which is a potent inhibitor of XO and is currently under investigation by the authors, is accounted for by these findings and successfully included in the model. This model incorporates many important insights into XO and can be of general interest. Moreover, it represents a clear-cut alternative to a previous model developed by Robins on the basis of the coordination of substrates and inhibitors to molybdenum.

Introduction

Xanthine oxidase (XO) is a very important enzyme in the purine metabolism that catalyzes the oxidation of hypoxanthine and xanthine to uric acid (Scheme 1).[1] A substantial amount of work has been done on XO in order to shed light on its mechanism of action: spectroscopic studies (EPR, Raman) have elucidated many of its structural features, and biochemical studies have led to a fair understanding of the mechanism of reaction of substrates with the enzyme.[2] However, the crystal structure of XO, which could provide a rationale for many of these findings and a clue to the design of drugs for this enzyme, has not yet been solved. Only very recently has the crystal structure of aldehyde oxidoreductase,[3] a protein showing 52% similarity in amino acid sequence with xanthine oxidase, been reported.

Xanthine oxidase is a fairly complicated enzyme. It is a 300 000 Da homodimer protein possessing two iron−sulfur centers and one flavin adenine dinucleotide (FAD) in addition to one molybdenum center in each of the subunits.[4] Xanthine and hypoxanthine are oxidized at the molybdenum center, the metal being reduced from the VI to the IV valence state; the reducing equivalents are transferred to molecular oxygen at the FAD with the mediation of the iron−sulfur centers.[5] The molybdenum center contains the MoOS functional unit, which is mainly responsible for the catalytic activity, and a pterin cofactor that coordinates molybdenum through its dithiolene side chain. Whether a direct Mo−C_8(xanthine) bond or a Mo−O−

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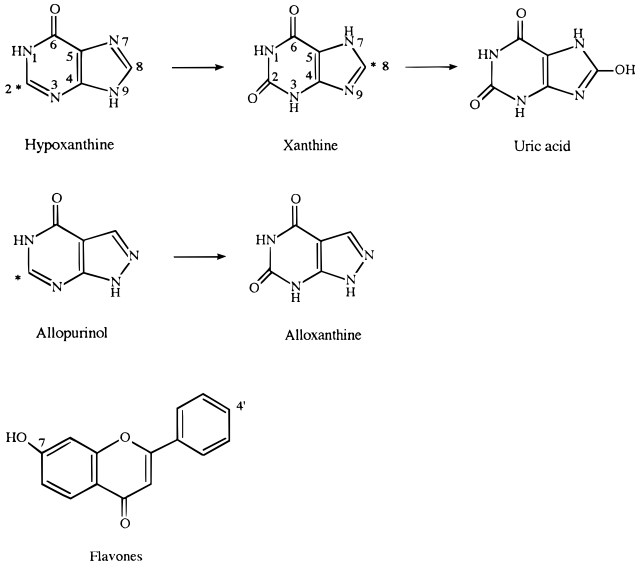
Scheme 1. Oxidation of Hypoxanthine, Xanthine, and Alloxanthine by Xanthine Oxidase[a]



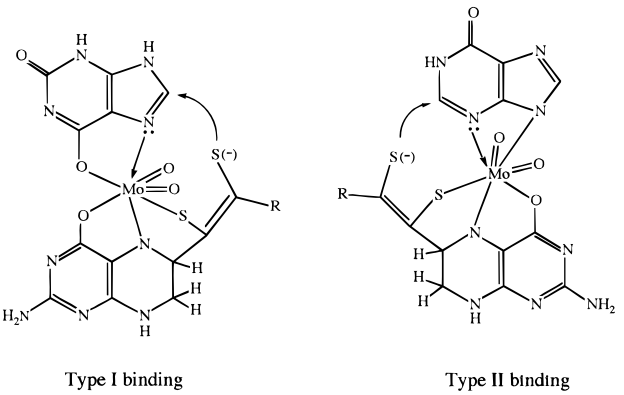
[a] The oxidized positions on the purine ring are marked with an asterisk. The structure of 7-hydroxyflavone, an inhibitor of the enzyme, is also reported.

C_8 bond is formed during catalysis is still a matter for debate, even though recent findings support the former mechanism.[6]

The inhibition of XO is important for two principal reasons: firstly, it decreases the production of excessive uric acid developed under hyperuricemic conditions that ultimately cause gout;[7] secondly, it prevents the formation of the superoxide radical, and is therefore important as a protection against post-ischemic reperfusion injury.[8] Allopurinol (Scheme 1) is currently the drug of choice for the treatment of hyperuricemia and gout.[9] It acts both as a substrate and as an inhibitor, being first oxidized to alloxanthine and then tightly bound by XO. However, allopurinol gives rise to certain severe adverse effects, such as leukopenia, dermatitis, rashes, renal impairment, and gastrointestinal disorders.[10] Moreover, both allopurinol and alloxanthine, owing to their purine-like structure, are known to undergo conversion to the corresponding nucleotides[11] and are apt to cause interactions with other enzymes involved in the purine metabolism.

Accordingly, we have recently undertaken a research program for the discovery and optimization of new XO inhibitors having structures not related to purine; our studies started with natural polyhydroxylated compounds, namely, flavones[12,13] and anthocyanidins,[14−17] and proceeded with the design and synthesis of a new series of 4′-substituted flavones which proved to be very potent inhibitors.[18] On interpretative grounds, these studies allowed us to conclude that flavones are active in the dissociated (anionic) form that originates from the proton dissociation of the C7-hydroxyl,[13] and that the substituents at the C4′-substitution site are mainly involved in dispersion interactions with an aromatic amino acidic residue of the enzyme.[18] An effective way of testing these proposals would be to carry out a protein crystallographic study of the structure of the complexes of flavones with XO; however, this would require that the crystal structure of XO had first been determined—something that has not yet been achieved. In the absence of direct information about the binding of ligands with XO, it is therefore necessary to devise models able to account for and interpret data, and to rely on these models for rational drug design.

Chart 1. Coordination of Molybdenum by Xanthine (type I) and Hypoxanthine (Type II) Proposed by Robins *et al.*[19,a]



[a] The chart also shows the coordination of the pteridine cofactor.

From the structure−activity data of an extended set of xanthine, hypoxanthine, and guanine analogs acting as inhibitors of XO, Robins *et al.*[19] have proposed a model of binding of these ligands with the molybdenum center. This model relies upon coordination to molybdenum and on the hypothesis that xanthine and hypoxanthine, the two physiological substrates of XO, coordinate molybdenum in two different ways (Chart 1, type I and type II binding), plus a third type of binding for alloxanthine (not shown).[19] However, more recent experimental findings appear to disprove these models, and the role in catalysis of a direct coordination of the substrates to molybdenum has also been questioned.[2] Therefore, it is timely to reconsider past and new evidence relating to the mechanism of XO, as well as new structure−activity data, with a view to formulating an alternative model of interaction of substrates and inhibitors with this enzyme.

This model will make use of theoretical tools such as quantum-chemical calculations with the inclusion of solvent effects, molecular superimposition with least-squares fitting algorithms, and molecular electrostatic potentials in order to identify the most important structural features of the substrates and inhibitors that interact with XO and to define geometrically the main regions in the binding site of the enzyme where the interactions take place. Special attention will be paid to xanthine and hypoxanthine, since these are the natural substrates of the enzyme; the relative stabilities of their tautomeric forms will be calculated in order to determine the most abundant form(s) present in solution; a superimposition of the structures of

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xanthine and hypoxanthine will then be proposed on the basis of the important role of the reactive centers and the carbonyls of these substrates, and the similarity of the electrostatic potentials of the two molecules will be used to suggest how the two substrates can be oriented in the molybdenum binding site. The new model of interaction will then be tested and further developed using a series of dimensional analogs of xanthine and hypoxanthine synthesized by Leonard *et al.*,[20,21] and hypotheses regarding the amino acidic residues interacting with the carbonyls of the substrates will be put forward.

In the second part of the work, the model of the substrates will be extended to the inhibitors of XO: to this end, the most interesting of the inhibitors developed by Robins will be reconsidered and accordingly superimposed on xanthine and hypoxanthine; this operation aims to unify the binding of substrates and inhibitors and will also provide a map of the active-site region more suitable for hydrophobic interactions. Finally, the flavone pharmacophore will be accounted for by these findings and included in the model.

Methods

Quantum-chemical calculations were performed with the AM1[22] method available in the MOPAC[23] program package; geometry optimization was performed with increased convergence criteria.

Quantum-chemical calculations with the inclusion of solvent effects were performed with the AMSOL[24] program, using the AM1 Hamiltonian and the SM2[25] model for water. Only single-point calculations were performed in water using the gas-phase optimized geometries.

The computation of the molecular electrostatic potentials was performed with MEPSIM.[26] They were calculated in the plane of the molecule (module MEPPLA) using a 0.1 Å step size grid; the electron density distributions necessary for the computation of MEP were obtained from a STO-3G *ab initio* wave function, using Gaussian92.[27] The graphical representation of the isopotential lines was obtained with UNIGRAPH.[28]

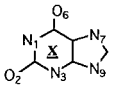
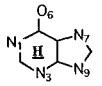
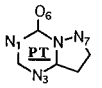
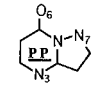
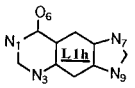
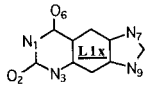
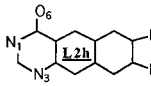
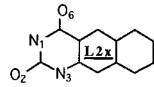
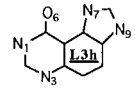
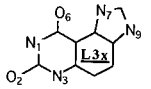
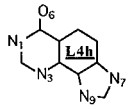
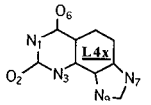
Molecular superimpositions with least-squares fit, as well as the graphical representations of molecules, were performed with MIDAS.[29]

All calculations were performed on a Convex C220 and molecular visualization on a Silicon Graphics Indigo2 at the Computing Center of the University of Modena (CICAIA).

The Substrates

Xanthine and Hypoxanthine. For our purposes, xanthine and hypoxanthine constitute the reference molecules on which to construct a model of interaction of substrates with XO. A systematic study of these two molecules was therefore undertaken; firstly, their geometries were completely optimized with AM1, taking into account the whole set of tautomeric forms in which covalent structures could be drawn. Table 1 reports the relative energies of the various tautomeric forms of xanthine (X) and hypoxanthine (H), and also of two pyrazolo inhibitors (PT and PP) and eight substrate analogs (L1−L4h,x), which will be discussed later. The most stable tautomers of both X and H have the N_1-H lactam form; X1 also has the stable N_3-H lactam form, unlike hypoxanthine, which lacks the O_2 carbonyl. These findings are true both *in vacuo* (AM1) and in water (AM1/SM2). The solvated lactim forms are more than 9 kcal/mol less stable than the lactam ones; the O_6-H lactim form of

Table 1. Relative Stabilities (kcal/mol) of the Various Tautomeric Forms of Xanthine and Hypoxanthine (Substrates), Two Pyrazolo Analogs (Inhibitors), and Several Analogs of Xanthine and Hypoxanthine (Substrates) *in Vacuo* (AM1) and in Water (AM1/SM2)[a]

		N_1	O_2	N_3	O_6	N_7	N_9	ΔE^{AM1}	$\Delta E^{AM1/SM2}$
	*X1*	H		H		H		0	0
	X2	H		H			H	4.2	2.2
	X3	H	H				H	10.9	9.0
	X4	H	H			H		12.0	10.8
	X5			H	H	H		13.9	11.3
	X6	H			H		H	16.1	14.3
	X7		H	H		H		17.6	13.8
	X8			H	H		H	18.1	16.3
	X9	H		H	H			19.1	17.8
	X10		H	H			H	24.6	17.4
	X11	H			H	H		27.1	23.8
	*H1*	H					H	0	0
	H2	H				H		0.9	2.4
	H3				H		H	3.8	8.8
	H4				H	H		6.1	9.0
	H5			H		H		7.0	5.1
	H6			H			H	14.0	8.5
	H7			H	H			17.6	15.0
	H8	H			H			20.3	15.4
	*PT1*	H						0	0
	PT2			H				6.1	2.7
	PT3				H			10.2	13.8
	PT4					H		15.7	15.3
	*PP1*			H				0	0
	PP2				H			2.3	7.1
	PP3					H		7.4	9.6
		H					H	0	0
		H				H		0.5	0.9
				H		H		9.5	6.0
				H			H	9.7	6.2
		H		H			H	0	0
		H		H		H		0.5	0.2
		H	H				H	11.3	10.1
				H	H		H	14.2	12.6
		H					H	0	0
		H				H		0.2	0.4
				H			H	9.0	5.5
				H		H		9.1	5.5
		H		H			H	0	0
		H		H		H		0.4	0.2
		H	H				H	12.1	11.0
				H	H		H	15.1	13.2
		H				H		0	0
		H					H	7.0	3.5
				H		H		8.7	4.5
				H			H	17.6	10.0
		H		H		H		0	0
		H		H			H	7.8	4.1
		H	H			H		11.3	10.2
				H	H	H		15.3	13.6
		H					H	0	0
		H				H		2.8	2.0
				H		H		8.4	5.2
				H			H	13.4	9.5
		H		H		H		0	0
		H		H			H	4.0	4.0
		H	H				H	12.0	10.9
				H	H	H		14.1	11.9

[a] X = xanthine; H = hypoxanthine; PT = pyrazolo[1,5*a*]-1,3,5-triazin-4-one; PP = pyrazolo[1,5*a*]-pyrimidin-7-one; L1−L4h,x are hypoxanthine (h) and xanthine (x) dimensional analogs (see Chart 2). For clarity, the numbering system of PT, PP, and L1−L4h,x was kept consistent with that of X and H.

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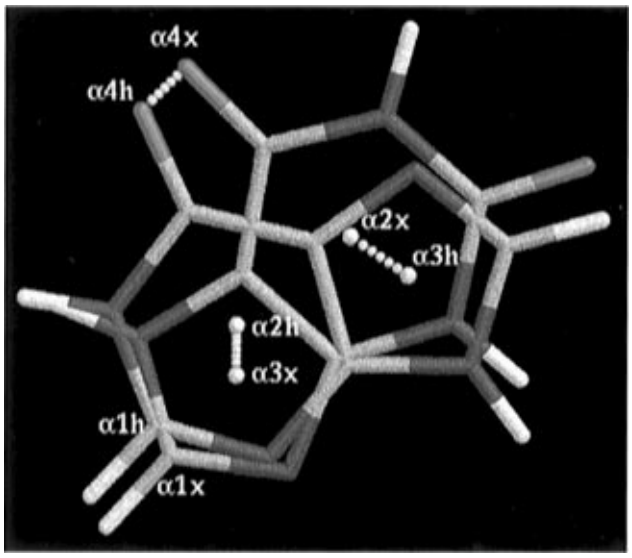

Figure 1. Superimposition of the structures of xanthine and hypoxanthine (red = oxygen, blue = nitrogen, white = hydrogen). The distances in angstroms between the equivalent centers resulting from the matching of the two substrates are α1h−α1x = 0.38, α2h−α3x = 0.57, α2x−α3h = 0.78, and α4h−α4x = 0.69.

hypoxanthine (H3) is only 3.8 kcal/mol less stable than H1 *in vacuo* but is strongly disfavored by solvent (8.8 kcal/mol). The only tautomeric forms whose values of ΔE are compatible with the presence of a certain amount in solution are the N_7-H and N_9-H ones (X1, X2, H1, H2), with a dominance of the N_7-H tautomer for xanthine (X1) and of N_9-H for hypoxanthine (H1).

Orientation of Xanthine and Hypoxanthine in the Active Site. Xanthine and hypoxanthine must enter the active site properly oriented to closely approach and react with the MoOS unit; since oxidations occur at the MoOS unit of XO, hypoxanthine must approach MoOS with its C_2 oxidable carbon atom, xanthine with its C_8 carbon atom (Scheme 1). This feature suggests that C_2 of hypoxanthine must be assumed to be geometrically equivalent with C_8 of xanthine (center α1); it also implies that the six-membered ring of one substrate is geometrically equivalent with the five-membered ring of the other, a condition that can be simply imposed by requiring the equivalence of the geometrical centers of the two rings (centers α2 and α3). Furthermore, the carbonyl oxygen O_6, which is common to the two substrates, has been taken as a fourth center (center α4) on account of its determinant role: in fact, there is a complete loss of catalytic activity in purine analogs in which the carbonyl group is replaced by a methyl (6-methylpurine) and a methoxyl (6-methoxypurine);[30,31] on the other hand, a carbonyl at the position corresponding to position 6 of hypoxanthine considerably increases the catalytic activity of heterocycles even different from purines, like pyrimidine, pyrazolo[3,4-*d*]pyrimidine, *v*-triazolo[4,5-*d*]pyrimidine, and pteridine.[30] The carbonyl in question is therefore crucial to activity. The finding that the same carbonyl is also essential for the activity of purine analogs acting as inhibitors[19] suggests that the carbonyl may function as a general anchoring and binding interaction site common to substrates and inhibitors.

Once α1−α4 had been defined, the best superimposition of these centers in xanthine and hypoxanthine was obtained using a least-squares fitting matching algorithm. The result is graphically reported in Figure 1 along with the distances in angstroms between these centers. This superimposition is satisfactory for several reasons: (i) the equivalent centers of xanthine and hypoxanthine are close together, particularly in the case of the reactive centers α1 and the important carbonyl centers α4; (ii) interestingly, the hydrogen bond donors and acceptors of xanthine and hypoxanthine turn out to be positioned in the same region; (iii) there is a good overlap of the van der Waal volumes of the two molecules; (iv) in this orientation, the electrostatic potentials of xanthine and hypoxanthine are very similar. The latter feature is noticeable because electrostatic interactions are the main long-range forces responsible for the recognition and relative orientation of ligands, so that a close similarity is assumed to be a requisite for a similar orientation. At a shorter range, the close similarity of the electrostatic potentials also implies similar hydrogen-bonding patterns of the ligands with the enzyme counterpart. This is particularly important when different tautomeric forms are to be considered, because they show strong differences both in the electrostatic potential shape and in the hydrogen-bonding pattern.

The electrostatic potentials calculated in the plane of the purine rings of the two more stable X1 and X2 tautomers of xanthine and H1 and H2 of hypoxanthine are shown in Figure 2, with the molecules already oriented as in Figure 1 in order to facilitate comparison; taking into account the minimum energy tautomers X1 and H1 first, a close similarity can be observed between the negative regions of the O_6 carbonyls (centers α4) of the two substrates as well as between the negative region of N_9 of xanthine and N_3 of hypoxanthine. The X2, less stable, tautomer of xanthine, having the N_9-H form, does not match the negative electrostatic potential of N_3 in hypoxanthine and creates a negative region near N_7 which is not present in hypoxanthine; it is therefore less similar to H1; on the other hand, a proton on N_3 of hypoxanthine, which would make hypoxanthine more similar to X2 in this region, inevitably involves the loss of the N_1-H proton that matches with the N_7-H proton of xanthine, and is also strongly disfavored in energy (Table 1). Whether H1 or H2 can be more readily superimposed on X1 is not clear; however, they both retain electrostatic complementarity with X1 in a determinant region of the catalytic site of the enzyme, *i.e.*, the one that faces the MoOS catalytic unit, while leaving the differences in a region that is probably exposed to the water molecules in the enzymatic cavity.

This model of interaction for xanthine and hypoxanthine is rather different from that originally proposed by Robins *et al.*,[19] who suggested that the two substrates afford coordination bonds with molybdenum in two different ways (Chart 1, type I and type II binding). Although a definitive statement cannot be made until more information is forthcoming from protein crystallography, the model of Chart 1 is open to criticism: (i) the notion that the covalent attack to C_8 and C_2 of the substrates is carried out by the dithiolenic sulfur of the pterin cofactor has been disproved by more recent experimental findings which demostrate that the attack occurs by means of the MoOS catalytic unit, the cofactor's role being simply in modulating the reactivity and/or the reduction potential of MoOS; (ii) the direct coordination of xanthine and hypoxanthine to molybdenum, and even in two different ways, is unlikely to play a role in catalysis.[2] Our model, on the other hand, is not based upon coordination, unifies the type of binding of xanthine and hypoxanthine, takes into account a superimposition of the two carbonyl oxygens of the substrates and, finally, is consistent with EPR studies of complexes of xanthine and 1-methylxanthine with XO;[32] from these studies it emerged that xanthine gives rise to two "rapid" EPR signals, attributed to two different

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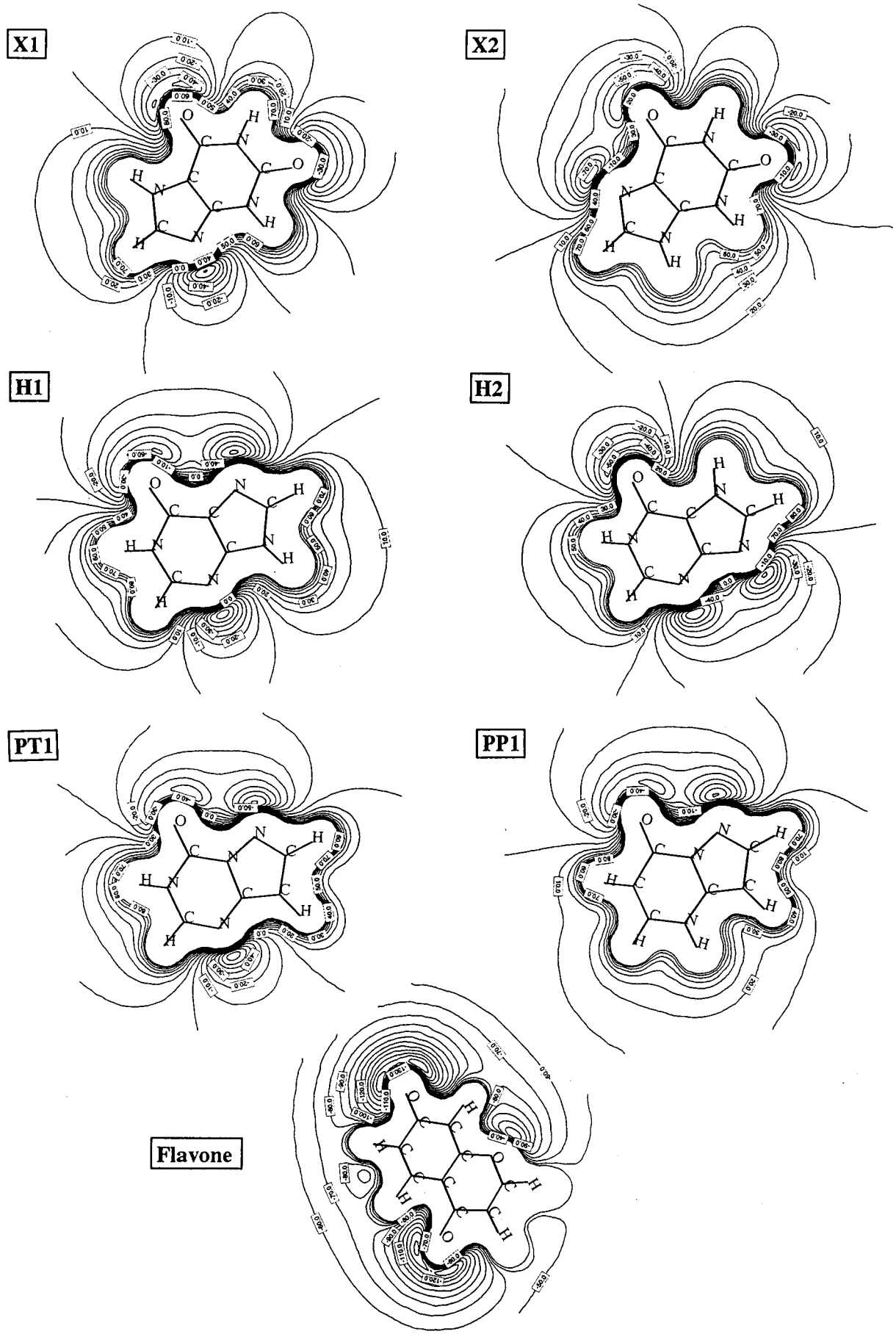
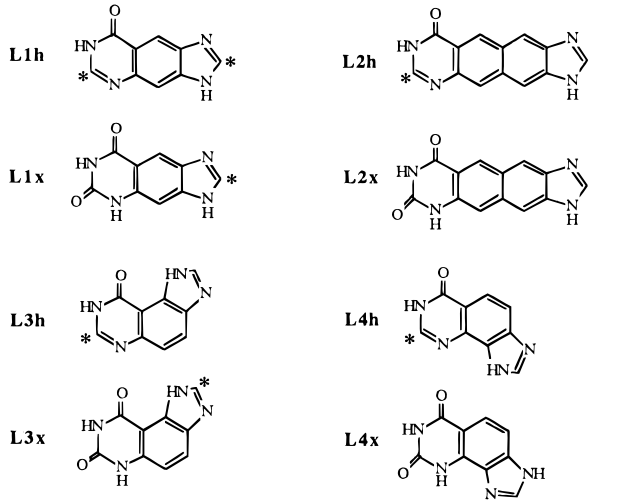


Figure 2. Electrostatic potentials (contour levels) of the most stable tautomers of xanthine (X1, X2) and hypoxanthine (H1, H2), as well as of the most stable tautomers of pyrazolo[1,5*a*]-1,3,5-triazin-4-one (PT1) and pyrazolo[1,5*a*]-pyrimidin-7-one (PP1). The electrostatic potential of the flavone pharmacophore, which is dissociated at the 7-hydroxyl group, is also reported. In order to facilitate the comparisons, the structures have been oriented as in Figure 1.

Chart 2. Dimensional Analogs of Hypoxanthine (L1h−L4h) and Xanthine (L1x−L4x) Synthesized by Leonard *et al.*[20,21,a]



[a] They were obtained by interposing one or two benzene rings, both linearly and angularly, between the two purine rings of hypoxanthine and xanthine. The positions still oxidized by XO are marked with asterisks.

orientations of the substrate with respect to the molybdenum center, and that only one of these leads to the final product. More recently, Hille and co-workers[33] reported that the substrate binding is a two-step process involving two different equilibrium constants, and they attributed these differences to two orientations of the substrate with respect to the molybdenum. We propose that the two orientations observed for xanthine could be due to the rotation by which its five-membered ring is exchanged for the six-membered ring (with respect to molybdenum), just as in the rotation that we use to superimpose xanthine on hypoxanthine in our model. Only the orientation whereby the five-membered ring of xanthine (*i.e.*, C_8) faces MoOS can in fact be productive. By way of confirmation, 1-methylxanthine gives only the productive EPR signal: the steric bulk of the methyl in the six-membered ring possibly prevents the formation of the complex in which this ring is orientated toward MoOS, but leaves the other orientation, which is indeed the productive one, unchanged.

Substrate Site Mapping. In general, chemical modifications to the functional groups of the six- or five-membered rings of xanthine and hypoxanthine that lie in proximity of MoOS, like methylation of the nitrogens or methoxylation of the carbonyl, lead to substantial decreases in catalytic activity. Therefore, a valid approach to the problem of mapping the dimensions of the substrate active site is the one proposed by Leonard *et al.*:[20,21] they extended the length, both linearly and angularly, of the purine rings of xanthine and hypoxanthine, leaving the structures of the fundamental peripheric rings intact (Chart 2, compounds L1−L4, h = hypoxanthine-like, x = xanthine-like). The finding that not all C_2 and C_8 positions could still be oxidized by XO (marked with an asterisk in Chart 2) is particularly interesting for our purposes, because it offers the possibility to probe and extend the model developed for xanthine and hypoxanthine.

Firstly, the relative energies of the tautomers of L1−L4h,x were calculated (Table 1). The results indicate that the linear interposition of one and two benzene rings in hypoxanthine (L1h, L2h) and in xanthine (L1x, L2x) leads to much more similar energies of the N_7 and N_9 tautomers (Table 1). Therefore, the linear analogs are almost free to assume the tautomeric form (N_7/N_9) more suited to the enzyme. On the contrary, the angular interposition of a benzene ring (L3h,x, L4h,x) gives neat preferences to the tautomer showing intramolecular hydrogen bonding. In L3h and L3x, the N_7-H tautomer is much more stable than the N_9-H one because of hydrogen bonding with O_6. Instead, the N_9-H tautomer is the preferred one for L4h because of hydrogen bonding with N_3, and the N_7-H tautomer is preferred for L4x because the N_9 nitrogen hydrogen bonds N_3-H.

Thus, compounds L1−L4x,h were superimposed on xanthine and hypoxanthine, oriented as in Figure 1, using the same matching criteria as before, *i.e.*, reactive centers, carbonyls, and geometric centers of the rings. The results are reported in Figure 3. Compound L1h (white) shows a perfect superimposition of its C_2 reactive center and of its carbonyl (marked with a red arrow) with xanthine and hypoxanthine, and is coherently active; on the contrary, the matching of L1x displays a strong deviation with considerable distances between the equivalent centers (L1x, match 1); in particular, the reactive center of L1x would be about 1 Å shifted toward MoOS with respect to xanthine and hypoxanthine, thus being in steric conflict; this situation occurs because the request to superimpose the more distant carbonyl of L1x is not compatible with a good superimposition of its reactive center. Since the reactive center is the most stringent condition to be satisfied for the substrates, we tried the limit condition in which the carbonyl is omitted from the matching request: a good superimposition of the reactive center is obtained (L1x, match 2) at the cost that the carbonyl is shifted about 3 Å from its optimal position. Intermediate orientations between match 1 and match 2 are plausible and could reduce somewhat the carbonyl shift. Since L1x can still be oxidized at C_8, we are forced to assume that the enzyme counterpart tolerates this shift (≤3 Å). Larger deviations, like that of compound L2x (6 Å), are not tolerated by the enzyme, for L2x is completely inactive. L2h, on the other hand, can be well superimposed and is therefore active. It is interesting to note that this comparison of L2h and L2x attributes the lack of activity of L2x only to a wrong positioning of the carbonyl and not to steric conflict due to the extended length of the two molecules, because they protrude in very similar directions and entity with respect to xanthine and hypoxanthine (Figure 3). A further indication that the position of the carbonyl is indeed a discriminant factor is that both L2h and L2x (as well as L1h and L1x) share the correct tautomeric preferences of xanthine and hypoxanthine in the region closer to the important MoOS center of the enzyme.

Compounds L3h and L4h can also be nicely superimposed on xanthine and hypoxanthine (Figure 3), with the imidazole ring once pointing toward the carbonyl (L3h), once pointing in the opposite direction (L4h). Compound L3x is very interesting because, despite being angular, it affords a good match of all the equivalent centers; in particular, it places the carbonyl in a region suitable for interaction, and it protrudes in a direction very similar to that of L3h. Worthy of note is that the strong preference for the N_7-H tautomer for this compound (Table 1) is in perfect accord with the proposed superimposition, where the N_9 nitrogen of L3x is in a position very similar to N_3 and N_9 of hypoxanthine and xanthine, respectively. No satisfactory superimposition is possible for the inactive L4x. Indeed, (i) the opposite angularity prevents a good superimposition of all equivalent centers (its reactive center would be shifted about 2 Å toward MoOS), (ii) omitting the geometric center of the six-membered ring of L4x from the request (L4x, match 1), the reactive center is still 1 Å apart and the region optimal for the carbonyl is hindered by a benzene ring, and (iii) omitting the

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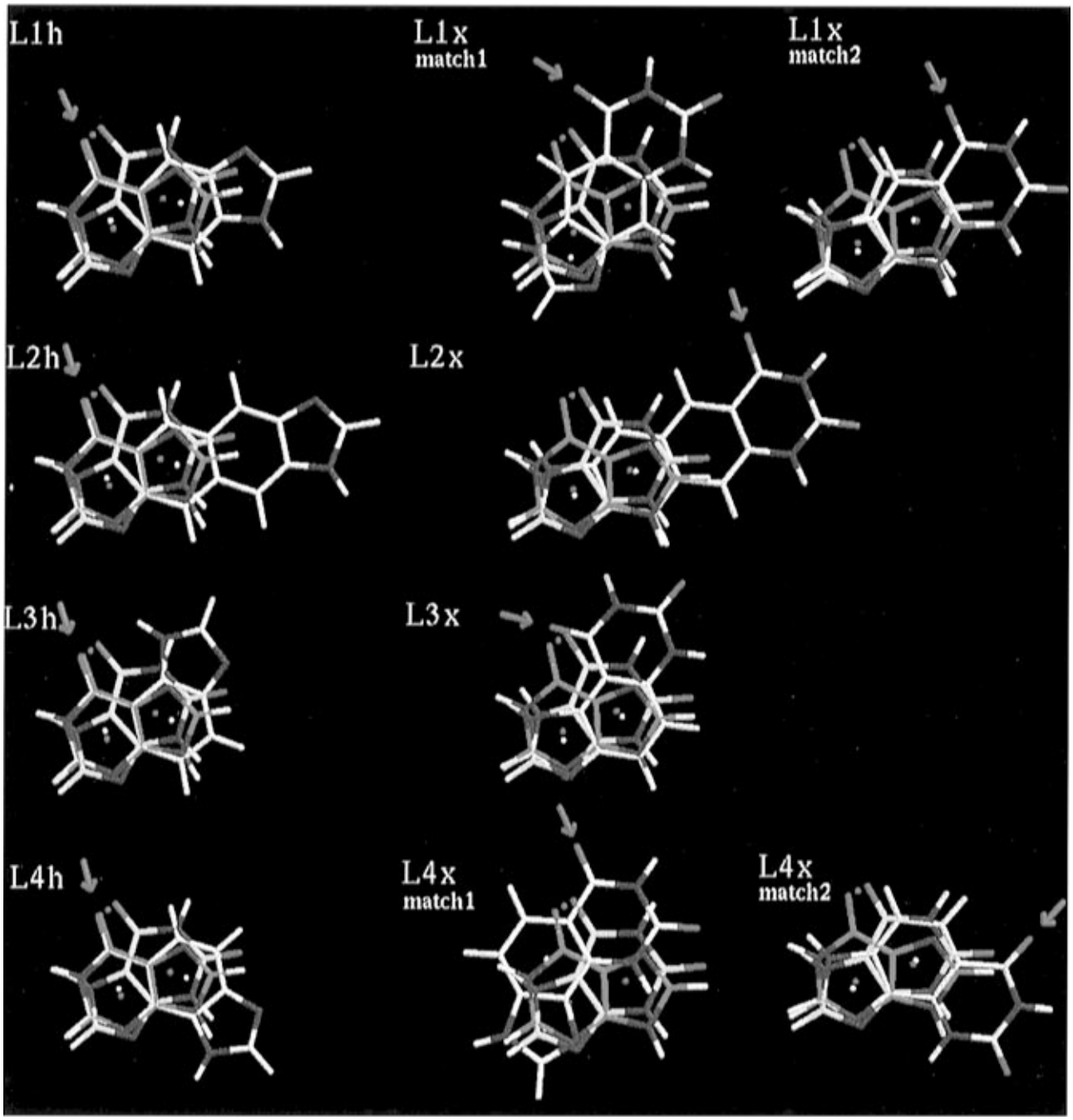

Figure 3. Superimposition of the dimensional analogs L1−L4 h,x (white) of Leonard *et al.* to hypoxanthine (cyan) and xanthine (yellow). The carbonyls are colored in red and identified with red arrows; the nitrogens are blue.

carbonyl request, the carbonyl itself falls in a region completely unsuitable for interactions (L4x, match 2).

Amino Acidic Residue Interacting with the Carbonyl. It turns out that a proper orientation of the carbonyl is very important for activity. Therefore, it is reasonable to suppose that the carbonyl interacts with a fundamental amino acidic residue of the active site. Two hypotheses can be formulated in the light of the information available in the literature. The first is that the carbonyl interacts with a lysine of the active site. Many findings support this hypothesis: the chemical modification of the lysine residues present in the active site of XO causes a complete loss of catalytic activity;[5] furthermore, many enzymes that use purines as substrates, like uricase,[34] hypoxanthine-guanine phosphoribosyltransferase,[35] and xanthine-guanine phosphoribosyltransferase[36] have in their sequences segments of amino acids in which a lysine is always conserved. In uricase, lysine is also fundamental for activity.[34] Finally, in the crystal structures of the complexes of purine-nucleoside phosphorylase with guanine[37] and that of hypoxanthine-guanine phosphoribosyltransferase with guanosine-5′-monophosphate,[38] the carbonyl oxygen of guanine just interacts with a lysine side chain. The second hypothesis is that the carbonyl interacts with a protonated glutamic acid side chain;

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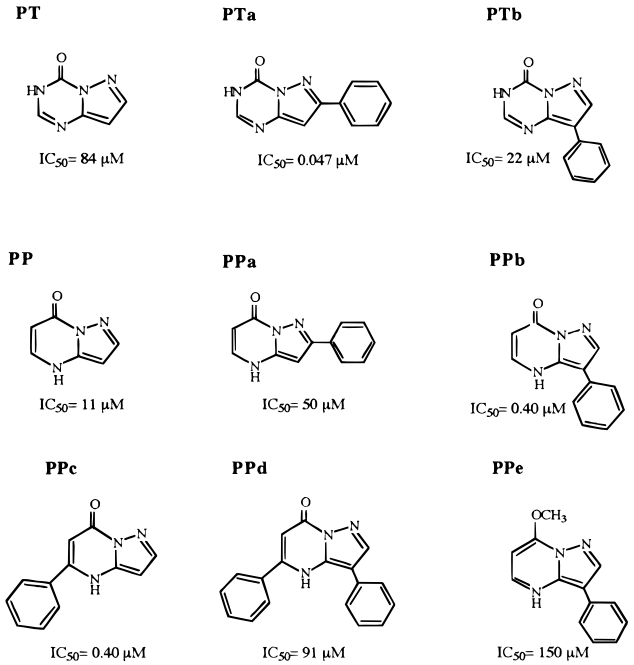
Chart 3. Chemical Structures and Inhibitory Activities of the Purine Analog Inhibitors Selected from the Work of Robins[19,a]



[a] PT and PP (pyrazolo[1,5*a*]-1,3,5-triazin-4-one and pyrazolo[1,5*a*]-pyrimidin-7-one, respectively) have been substituted with phenyl rings.

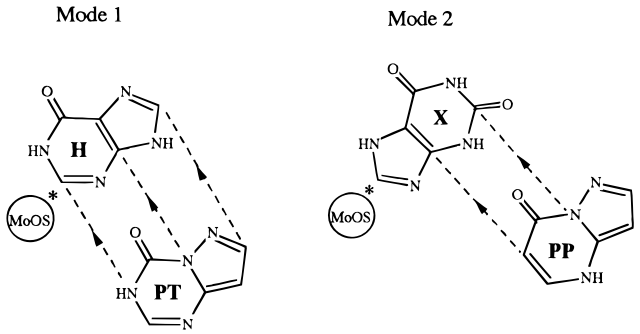
the evidence in favor of this hypothesis is that in the crystal structure of the xanthine oxidase-related aldehyde reductase[3] a protonated glutamic acid (Glu869) is in close proximity to the MoOS center and is totally conserved in the xanthine oxidase family. The suggestion that the glutamic acid side chain is protonated[3] is in perfect accord with our model because it enables an attractive interaction with the carbonyl group.

The residue interacting with the carbonyl being either a lysine or a protonated glutamic acid, the flexibility of their side chains could be invoked to explain why the 3 Å shift of the carbonyl of L1x (Figure 3) can be "tolerated" by the enzyme. In fact, it is logical to assume mutual conformational changes when slightly different positionings of interacting groups have to be overcome; however, it is very likely that the flexibility is not enough to reach the 6 Å-shifted carbonyl of L2x, which was in fact inactive.

Extension of the Model to the Inhibitors

Purine Analogs. Robins *et al.* have reported an extensive set of purine analogs acting as inhibitors of xanthine oxidase[19] in which the pyrazolo[1,5*a*]-1,3,5-triazin-4-one (PT, Table 1) and the pyrazolo[1,5*a*]-pyrimidin-7-one (PP, Table 1) emerge as the most promising purine nuclei. An important observation was that the introduction of a phenyl substituent on these nuclei considerably increased inhibitory activity, a finding that suggests the presence of a hydrophobic site in the enzyme. It is therefore of interest to define the optimal location of the phenyl rings of these inhibitors with respect to the map of the substrates. Chart 3 reports the most significant terms of the series together with their inhibitory activities (IC_{50}). Phenyl substitution is more effective at position 8 for nucleus PT (compare the activities of PTa *vs* PTb) and at position 9 for PP (PPb *vs* PPa). The substitution of a methoxyl for the carbonyl (PPe vs PPb) causes a complete loss of inhibitory activity, in agreement with what has already been observed for the substrates. This feature constitutes the first link between the model of interaction of

Chart 4. Two Modes of Superimposing the Purine Analog Inhibitors to the Model[a]



[a] The comparison of the electrostatic potentials of the inhibitors PT and PP with those of hypoxanthine (H) and xanthine (X) strongly suggests mode 1 for PT and mode 2 for PP. H and X have been drawn oriented as in Figure 1 (the orientation of the two substrates with respect to MoOS).

the substrates and that of the inhibitors, and the carbonyl of PP and PT can be consistently superimposed on those of X and H.

PT and PP show various tautomeric forms whose relative energies are included in Table 1. The most stable tautomer of PT (PT1) is very similar in structure to the most stable tautomer of hypoxanthine (H1) and also shows a strictly similar hydrogen bonding pattern; this is confirmed by the strong similarity of their electrostatic potentials (H1 and PT1 in Figure 2). Therefore, compounds PT can be directly superimposed on hypoxanthine, *i.e.*, their six- and five-membered rings can be superimposed to the six- and five-membered rings of hypoxanthine, respectively (Chart 4, mode1). On the other hand, the most stable tautomer of PP (PP1) bears a protonated nitrogen at N_3, and cannot match the negative electrostatic potential of xanthine N_9 and hypoxanthine N_3 (X1, H1, and PP1 in Figure 2); tautomer PP2, which has the required negative N_3, is 7 kcal/mol less stable that PP1 (Table 1) and bears a hydroxyl at position 6 instead of a carbonyl; tautomer PP3, although bearing the negative N_3 and a carbonyl at position 6, is 10 kcal/mol less stable. In spite of these unfavorable features, compounds PP are still active, so that there ought to be a way to superimpose their structures to the model. One possibility, suggested by the comparison of the electrostatic potentials of PP1 and xanthine, is to superimpose the six-membered ring of PP1 to the six-membered ring of xanthine (Chart 4, mode 2). Thereby, the electrostatic similarity in the region facing MoOS can still be respected (Figure 2), and the inhibitors PP would be about 2 Å more distant from MoOS compared to X, H, and PT. Recall that the request to superimpose the reactive centers of xanthine and hypoxanthine in the region close to MoOS was a strict condition for the substrates (see the substrate section), but not necessarily for the inhibitors, which bind XO without undergoing oxidation by MoOS; therefore, a slight displacement of PP from MoOS is plausible, provided that the carbonyl is correctly positioned and the electrostatic potentials match.

For a definition of the region more suited to hydrophobic interactions, the phenyl derivatives of PT and PP (PTa,b and PPa,b) have been superimposed to the model using mode 1 and mode 2, respectively. The results are reported in Figure 4, where the positions corresponding to the most active phenyl substituents are colored in red. It is noteworthy that, using the templates of Chart 4, the most active compounds, PTa and PPb, locate the phenyl substituents in a very similar region of the active site (the red rings in the lower part of Figure 4), despite the fact that the phenyls are now at position 9 and position 8 of the purine nuclei.

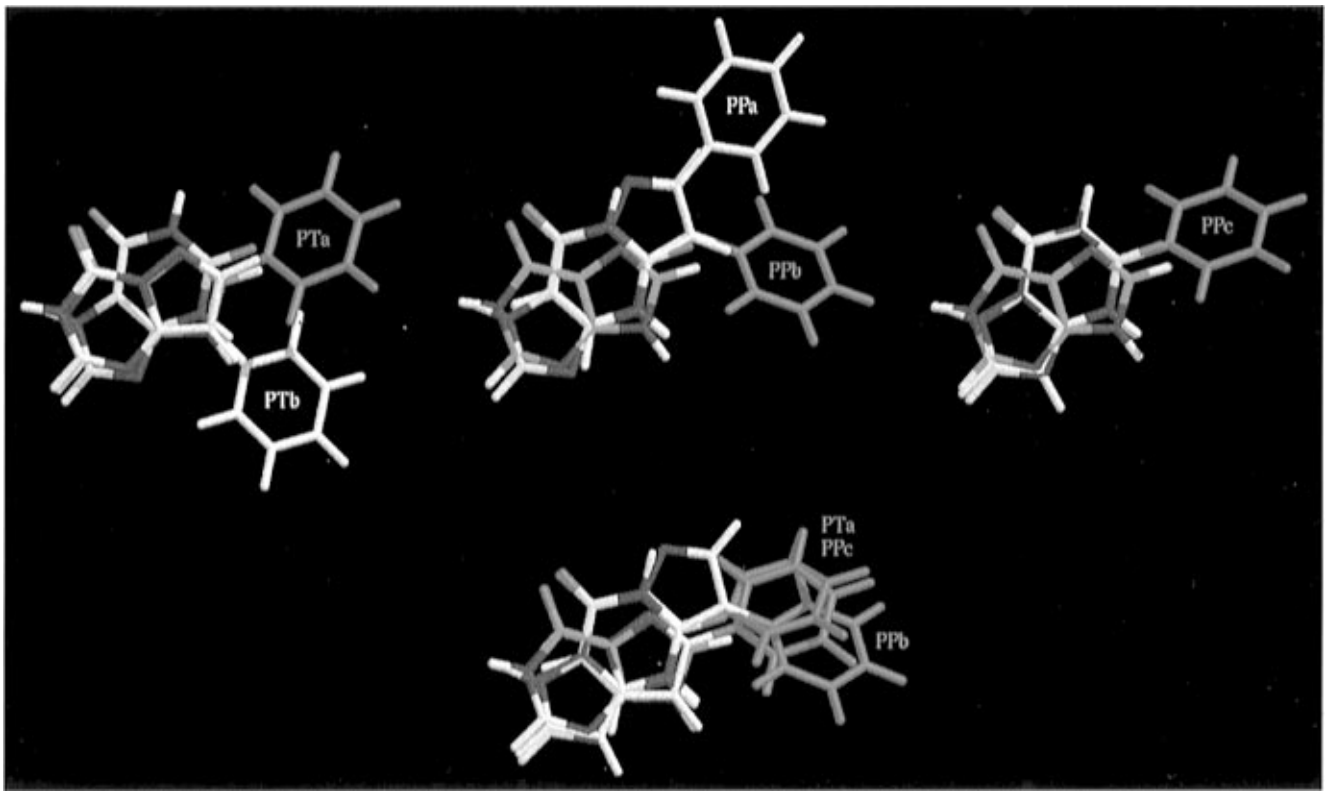


Figure 4. Superimposition of the phenyl-substituted derivatives of PT (PTa and PTb) and PP (PPa, PPb, and PPc) with xanthine and hypoxanthine. The regions occupied by the phenyl rings of the more active derivatives are colored in red, and are the more suitable to hydrophobic interactions with the enzyme. In the lower part of the figure, the global superimposition of the more active derivatives (red) shows that their phenyl rings occupy very similar positions despite being in different positions on the purine ring.

Similar arguments can also be used to explain why PPc is still active despite having a phenyl at position 2. In the case of PPc, a superimposition using mode 2 is not possible because of steric repulsion of the phenyl ring with the MoOS functional unit. On the other hand, PPc can be rotated and nicely superimposed on xanthine with its five-membered ring facing MoOS (Figure 4), with the result that the phenyl ring can still occupy the optimal red region. This is one more example in which the six- and five-membered rings can be interchanged. The strongest evidence in support of the exchange proposed for PPc is that compound PPd is not active: in fact, since PPd has both phenyl rings at positions 9 and 2, it is able to assume neither the orientation of PPb nor that of PPc.

Flavones. Flavones are a very interesting class of xanthine oxidase inhibitors. We recently showed that flavones are active in the dissociated (anionic) form that originates from the dissociation of the C7-hydroxyl (Scheme 1).[13] Furthermore, the 4′-substitution site was found to be very important in modulating inhibitory activity, substituents in this position being mainly involved in dispersion interactions with the enzyme.[18] It is now of interest to place the flavone pharmacophore in the model of interaction here proposed.

The first link between flavones and the model is that the anionic oxygen at C-7, being fundamental for activity, must be superimposed on the carbonyl oxygen of the purines; indeed, the anionic oxygen of the flavone pharmacophore has a very strong carbonyl character because of the extended delocalization of the negative charge on the entire benzopyrone ring. The electrostatic potential of flavone in its anionic form (Figure 2) is rather different from that of purines, mainly because of the negative charge. However, it is reasonable to try to superimpose flavone using mode 2 as a template. The result is reported in Figure 5; a good similarity with purines can be observed: the region of negative electrostatic potential of O_7 matches the negative region of the carbonyls of xanthine and hypoxanthine, and one of the lone pair minima of O_4 of flavone approaches the negative regions of N_3 of hypoxanthine and N_9 of xanthine (Figure 2); of special relevance is that the superimposition of the carbonyl oxygen, an essential requisite for activity, also achieves a good superimposition of the 2-phenyl ring of flavones with the phenyl rings of the most active purine inhibitors PTa, PPb, and PPc (Figure 5). This finding enables us to conclude that the 2-phenyl substituent of flavones is involved in hydrophobic interactions with the enzyme in the same region of the purine analog inhibitors. The finding that the 3-phenyl isomers of flavones are considerably less active than the 2-phenyl ones[39] stresses the importance of a correct positioning of the phenyl ring in the case of flavones as well. Moreover, a recent finding of ours that an additional phenyl substituent at position 4′ of 7-hydroxyflavones considerably enhances inhibitory activity[18] suggests that the hydrophobic region suitable for interaction with inhibitors can be successfully extended over the red area of Figure 5.

Position 7 of flavones is therefore strategic: it ensures a rather low pK_a of the hydroxyl (and thus a certain amount of dissociated form at physiological pH) due to a substantial delocalization of the negative charge, and at the same time, it enables flavones to give hydrophobic interactions in the optimal region of the enzyme. Let us recall that the hypothesis of a lysine residue interacting with the carbonyl of the substrates (see the substrate section) is particularly appealing in the case of flavones because of the strong electrostatic interaction between the anionic flavone and the positive lysine side chain.

Concluding Remarks

We have presented a model of interaction of substrates and inhibitors with xanthine oxidase that is based on, and is able to

(39) Costantino, L.; Rastelli, G.; Albasini, A. Unpublished results.

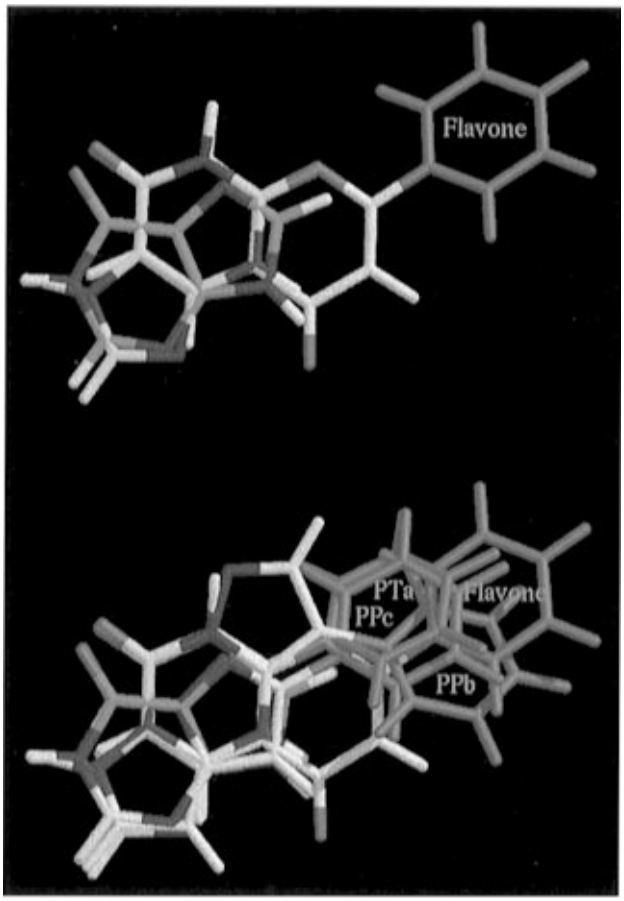


Figure 5. Superimposition of flavone to xanthine and hypoxanthine. In the lower part of the figure, the global superimposition of the most active derivatives of Figure 4 and flavone (red area) shows that the 2-phenyl ring of flavone lies very close to the phenyl rings of the other purine derivatives.

account for, many experimental insights into the mechanism and structure of the enzyme. Since the crystal structure of XO is not yet known, the relative orientation of the physiological substrates, xanthine and hypoxanthine, with respect to the molybdenum center of the enzyme is inferred by a superimposition of the most important functional groups of the substrates and validated by the similarity of their electrostatic potentials. The results show that the two substrates can achieve a high degree of similarity when oriented according to a rotation that exchanges the five-membered for the six-membered ring. This rotation is consistent with many experimental findings and, in particular, takes into account the importance of a carbonyl group for the catalytic activity of the substrates by explicitly requiring their superimposition. The superimposition of several dimensional analogs of xanthine and hypoxanthine allows us to confirm the importance of a correct positioning of the carbonyl for a suitable interaction with XO and to map the dimensions of the active site with respect to the orientation of xanthine and hypoxanthine here proposed. Then, on the basis of biochemical and structural evidences on XO and related enzymes, we suggest that the carbonyl oxygen interacts either with a lysine residue or with a protonated glutamic acid present in the active site of the enzyme.

The extension of the model to the most interesting inhibitors previously developed by Robins *et al.* gives very interesting results. Firstly, it is able to unite the binding of substrates and inhibitors to xanthine oxidase in a single model. Secondly, it is able to rationalize the inhibitory activities of the phenyl-substituted purines and to identify the most suitable location of the phenyl rings with respect to the map of the substrates. An important result is that the model is consistent with a unique location of the phenyl rings, even though the latter are in completely different positions on the purine ring. Intriguingly, the model also holds for inhibitors that are not analogs of purines: for example, the flavone pharmacophore, which is a very promising lead for the discovery of new inhibitors of this enzyme, is successfully accounted for in the model, since we were able to superimpose its anionic oxygen at position C7 on the carbonyls of the substrates and inhibitors and its 2-phenyl ring on the phenyl rings of the most active purine inhibitors here considered.

To conclude, the model is simple and able to rationalize the behavior of the substrates and inhibitors in question. It is firmly based on experimental data relating to the mechanism and structure of the enzyme and can be a clear-cut alternative to a model previously developed by Robins. Obviously, its validation awaits proper experimental investigation in the course of future studies.

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