J. Enzyme Inhibition, 1998, Vol. 13, pp. 347–359
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INHIBITION OF ZINC METALLOPEPTIDASES BY FLAVONOIDS AND RELATED PHENOLIC COMPOUNDS: STRUCTURE–ACTIVITY RELATIONSHIPS

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(Received 11 November 1997; In final form 5 March 1998)

Flavonoids and other benzopyrone substances, having an appropriate hydroxylation profile, may inhibit the metalloenzymes leucine aminopeptidase (LAP), aminopeptidase M (AP-M), and carboxypeptidase A (CP-A). A structural feature that evidently favours the interaction between flavonoids and the three metalloenzymes is the 2,3-double bond conjugating the A and B rings and conferring a planar structure. This can be considered virtually indispensable for inhibition of the three metallopeptidases, though the hydroxylation profile required differed for each of the enzymes, and the interaction mechanism and behaviour also differed. The inhibitory effect of flavonoids on LAP was reversible, and to be effective the flavonoid had to have conjugated A and B rings and *ortho*-dihydroxylation on at least one of the aromatic rings. This same requirement was essential for inhibition by coumarins and was attributed to a catechol-like mechanism of interaction. The inhibitory effects on AP-M were due to inactivation of the enzyme, irreversibly altered by flavonoids with a 2,3-double bond and a minimum of one hydroxyl substituent on each of the aromatic rings. With CP-A, conjugation of the A and B rings enhanced the inhibitory effect of flavonoids, though it was not strictly required. The interaction between the polyphenolic substances tested and the two zinc aminopeptidases was not reversed by adding zinc to the reaction medium, indicating that the inhibition is not due to the coordination of the phenolic hydroxyl groups with the catalytical zinc of active site, though the presence of zinc affected the interaction behaviour differently according to each substance's hydroxylation profile.

Keywords: Flavonoids; Quercetin; Coumarins; Leucine aminopeptidase inhibitors; Aminopeptidase M inhibitors; Carboxypeptidase A inhibitors

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INTRODUCTION

Zinc metalloproteases are a family of enzymes widely distributed in bacteria, yeast, plant and animal tissues, which are involved in diverse processes being critical for life, embryonic development, bone formation, tetanus and botulism toxins, reproduction, arthritis and cancer.[1] Zinc aminopeptidases catalyse the hydrolysis of amino acid residues from the amino terminus of peptide substrates, and are of critical biological and medical importance because of their key role in protein modification and degradation and in the metabolism of a wide range of biologically active peptides. They play an important role in a variety of disease states, including cardiovascular diseases, inflammation, and metastasis.[2]

Leucine aminopeptidase (LAP: cytosol aminopeptidase, EC 3.4.11.1) is a hexameric enzyme with two zinc ions per protomer.[3] Increased levels of this enzyme are present in the serum of patients with various types of cancers i.e. pancreatic, breast, gastrointestinal, and pulmonary with hepatic metastasis[4] and LAP has been put forward as a tumor marker in testicular[5] and breast[6] cancer.

Aminopeptidase M (AP-M: membrane aminopeptidase EC 3.4.11.2) is a high-molecular-weight homodimeric enzyme containing one zinc ion per subunit.[7] This enzyme together with other proteases are associated with the proliferation and invasive growth of basal cell carcinomas[8] and epithelial skin tumours.[9] AP-M is involved in the degradation of extended opioid peptides and is important in cardiovascular regulation, hypothermia, and analgesia,[10] as well as in the immunoresponse of T-cells.[11]

These enzymes have long been used to diagnose various physiological states and disease conditions and have furnished interesting molecular targets for the development of new drugs,[12] and the pharmacological activity of certain drugs may also be associated with their potential ability to interact with these enzymes.

Flavonoids are benzo-γ-pyrone derivatives widely distributed throughout the plant kingdom, contributing much of the colour of the component aerial parts, and therefore important constituents of the human diet. Although flavonoids generally have been considered to be non-nutritive agents, an important spectrum of biological activities are displayed by this group of substances. The antioxidant and free radical scavenging properties of plant polyphenols linked with their ability to coordinate metal ions and to interact with different enzymatic systems are the main causes for their beneficial effects on human health.[13]

Flavonoids have revealed their capacity to interact with different enzyme systems critically involved in pathological processes, and specifically certain

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flavonoid compounds have shown LAP inhibition,[14] but data is scarce about their interactions with other zinc metallopeptidases. The object of the present study was to investigate and compare the interaction behaviour of twenty five polyphenolic compounds, flavonoids and related structures, with two zinc aminopeptidases, LAP and AP-M and with a zinc carboxy-exopeptidase, carboxypeptidase A (CP-A: EC 3.4.17.1). The final purpose of our study is to ascertain the possible roles of these phytochemicals in controlling the processes mediated by these zinc metalloenzymes and to establish structure–activity relationships.

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MATERIALS AND METHODS

(A) Chemicals

(−)-Epicatechin, apigenin, luteolin, 3′,4′-dihydroxyflavone, 6,7-dihydroxy flavone, 7,8-dihydroxyflavone, kaempferol, fisetin, rhamnetin, rutin, myricetin, eriodictyol, 3,4-dihydroxycoumarin, dicoumarol, and mangiferin were from Extrasynthèse (Genay, France); morin from Merck; quercetin, taxifolin, 4-hydroxycoumarin, 7-dihydroxycoumarin, esculetin, 6,7-dihydroxy 4-methylcoumarin, scopoletin, and caffeic acid from Sigma Chemical Co. 5-Hydroxy-3,7,8,3′,4′-pentamethoxyflavone was kindly supplied by BBCAT (University of Alcalá). Structures of these compounds are presented in Table I. Compounds were dissolved in MeOH, except apigenin where EtOH was used.

(B) Enzymes and Substrates

Cytosolic LAP type V from porcine kidney (EC 3.4.11.1), AP-M from porcine kidney microsomes (EC 3.3.11.2), and CP-A type II from bovine pancreas (EC 3.4.17.1) were purchased from Sigma Chemical Co. L-leucine-*p*-nitroanilide (Sigma) was the substrate for both the aminopeptidases and hippuryl-L-phenylalanine (Sigma) for the carboxypeptidase A.

(C) Enzyme Assays

The rates of hydrolysis of L-leucine-*p*-nitroanilide by LAP and AP-M were assayed on microplates with 96 wells each at 25°C. A total volume of 210 μL of the incubation mixture was placed in each well. A 150 μL of 0.2 M triethanolamine–HCl buffer (pH 7.8 for LAP and pH 7.5 for AP-M), 25 μL of enzyme solution (LAP: 71 µg/mL; AP-M: 0.3 µg/mL), and 10 μL of a

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methanol (or ethanol) solution of each test compound, were preincubated for 10 min. The reaction was then initiated by adding 25 μL of 0.6 mM of substrate. A control was conducted in the absence of the test compound but with methanol or ethanol. *p*-Nitroaniline released by the substrate was continuously monitored at 405 nm for 30 min in a BIO-RAD microplate reader. The hydrolysis rate was estimated by simple linear regression.

CP-A activity was assayed according to Cannell *et al.*[15] A 0.3 mL of 1 M TRIS–HCl buffer, pH 7.5, 0.35 mL of a solution of each test substance in methanol : water (10 : 25, v/v) and 0.25 mL of CP-A (19 μM in 10% LiCl) were placed in test tubes. Reactions were started by adding 0.75 mL of 2 mM of substrate, hippuryl-L-phenylalanine, in 1 M TRIS–HCl buffer, pH 7.5. The test tubes were vigorously mixed and incubated at 37°C for 40 min. The enzyme reaction was then quenched by adding 0.1 mL of a methanolic solution of 10 mM of 1,10-phenanthroline. The enzymatic activity was calculated after quantification of the hippuric acid released by HPLC.

Chromatographic analyses were performed using a Hewlett-Packard (HP) model 1050 HPLC system with a 20-μL injection loop and an HP model 1040 M photodiode array detector coupled to an HP model 9000/300 personal computer and an HP model 9153 C disk drive.

A stainless-steel precolumn (150 × 3.9 mm I.D.) (particle size: 5 μm) (Waters) and column (250 × 4 mm I.D.) packed with Nucleosil 120 C18 (particle size: 4 μm) (Scharlau) were used. The mobile phase, 11% acetonitrile in 0.026 M acetate buffer (pH 4.0), was pumped in isocratic mode at 1.25 mL min^{-1}. Each sample contained paracetamol as internal standard at a concentration of 1.42 mM. Detection of hippuric acid was at 254 nm.[16]

Under these conditions the parameters of specificity, selectivity, linearity, accuracy and precision were verified in order to validate the method.

(D) Enzyme–Inhibitor Binding

To ascertain whether the inhibitors bound reversibly to the enzymes to form a complex, a mixture of enzyme and inhibitor was preincubated at 37°C for 30 min, and then ultrafiltered by Centricon-10 devices (Amicon), according to the methodology previously described.[17]

(E) Kinetic Studies

Kinetic studies employed the same methods described above, four different substrate and inhibitor concentrations being used. Inhibition mechanisms were graphically analysed by Lineweaver–Burk plots. IC_{50} values were

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estimated by non-linear regression analysis according to the logistic equation.[14]

RESULTS

Previous work carried out in our laboratory has described the use of *p*-nitroanilide derivatives as substrates in studies of the effects of flavonoids and related phenolic compounds on proteases, including LAP and has established the experimental conditions under which such compounds do not interfere with determinations of enzyme activity.[14,17] In the present study we have revalidated the assay conditions for LAP using microplates and established a protocol for AP-M. Table I presents the results obtained for the 25 substances tested on the two zinc amino exopeptidases, LAP and AP-M.

The activity of both enzymes was affected by the presence of certain compounds, mainly polyhydroxylated derivatives of benzochromone. These are metal chelators[18] and therefore could be able to alter the activity of these metalloenzymes by acting on the zinc atom involved in substrate hydrolysis. Should interaction involve the zinc ion, adding a solution of $ZnCl_2$ to the reaction medium should reverse the inhibitory effect.[19]

The findings (Table II) did not support interaction with the active site zinc and based on the spectral effects observed on adding a zinc solution to the compound (Figure 1), inhibition instead involved the structure of the phenolic compounds and the potential sites available for coordination with zinc.

The susceptibility of LAP and AP-M to the effects of the phenolic compounds differed. The study of the inhibition kinetics of active compounds on LAP showed that different mechanisms were involved (Figure 2).

Depending on the enzyme, enzyme inhibition was in certain cases reversed by ultrafiltration of the inhibitor–enzyme mixture (Table III).

In view of the different interactions between the polyphenolic substances and these two exopeptidases, the study was expanded to include another zinc metalloenzyme, CP-A, prototype of the carboxypeptidase group of enzymes[1] catalysing the hydrolysis of peptides or ester bonds at the C-terminus.

The enzymatic activity of CP-A was determined by HPLC, using hippuryl-L-phenylalanine as substrate and measured the hippuric acid released. Direct spectrophotometric determination at 254 nm[15] was not used, because at that wavelength the molar absorptions of hippuric acid and compound were very similar and, additionally, this method was also prone to interference by polyphenolic compounds. Under the conditions of separation

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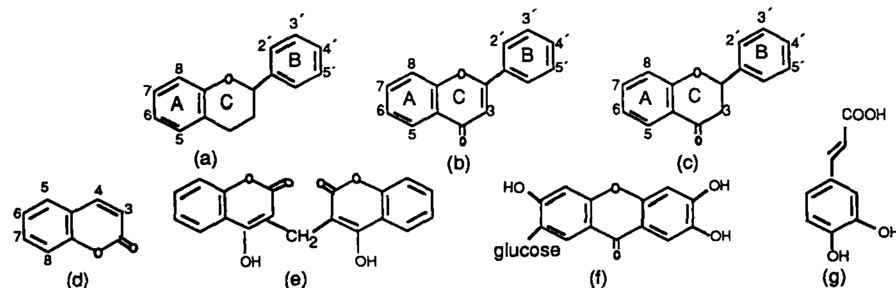
TABLE I Structures of the substances tested and their effects on leucine aminopeptidase (LAP) and on aminopeptidase M (AP-M) at 50 and 100 μM, respectively



Group	No.	Name	*Substitutions other than H or H_2 in the following positions of the reference structure (see footnotes)*									*Inhibition (%)***	
			2′	3′	4′	3	4	5	6	7	8	*LAP*	*AP-M*
(a)	**1**	Epicatechin		OH	OH	OH		OH		OH		14(±3)	—
(b) Flavones	**2**	Apigenin			OH			OH		OH		—	26(±10)
	3	Luteolin		OH	OH			OH		OH		62(±2)	11(±5)
	4	3′,4′-OH flavone		OH	OH							80(±4)	—
	5	6,7-OH flavone							OH	OH		9(±5)	—
	6	7,8-OH flavone								OH	OH	44(±6)	—
	7	5-OH 3,7,8,3′,4′-MeO flavone		MeO	MeO	MeO		OH		MeO	MeO	—	—
(c) Flavonols	**8**	Kaempferol			OH	OH		OH		OH		—	20(±4)
	9	Quercetin		OH	OH	OH		OH		OH		46(±7)	35(±8)
	10	Fisetin		OH	OH	OH				OH		56(±2)	25(±5)
	11	Rhamnetin		OH	OH	OH		OH		MeO		—	18(±5)
	12	Morin	OH		OH	OH		OH		OH		—	26(±6)
	13	Myricetin	OH	OH	OH	OH		OH		OH		—	25(±7)

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	14	Rutin	OH	OH	OX*		OH		OH	10(±2)	—
	15	Eriodictyol	OH	OH			OH		OH	—	—
	16	Taxifolin	OH	OH	OH		OH		OH	—	—
(d) Coumarins	**17**	4-OH coumarin				OH				13(±4)	—
	18	7-OH coumarin							OH	—	—
	19	3,4-OH coumarin			OH	OH				—	—
	20	Esculetin						OH	OH	70(±5)	13(±7)
	21	6,7-OH, 4-methyl coumarin				Me		OH	OH	30(±3)	—
	22	Scopoletin						MeO	OH	10(±5)	—
(e)	**23**	Dicumarol								—	—
(f)	**24**	Mangiferin								10(±2)	26(±3)
(g)	**25**	Caffeic acid								—	15(±5)

* OX: rutinose; ** $n=4$; —: no inhibition or less than 10%.

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TABLE II Effect of added $ZnCl_2$ on the inhibitory activity of the compounds

No.	Compound	*LAP-inhibition (%)**			*AP-M-inhibition (%)**			
		Conc. (μM)	*Without* $ZnCl_2$	0.1 mM $ZnCl_2$	*Conc.* (μM)	*Without* $ZnCl_2$	0.1 mM $ZnCl_2$	1 mM $ZnCl_2$
2	Apigenin	**			50	27.5(±8)	25.4(±7)	16.6(±5)
3	Luteolin	50	61.2(±2)	57.5(±2)				
4	3′,4′-dihydroxy flavone	50	79.4(±3)	70.4(±2)	**			
6	7,8-dihydroxy flavone	30	21.7(±10)	7.4(±5)	60	7.0(±2)	19.3(±7)	
9	Quercetin	50	45.6(±6)	62.5(±5)	60	33.2(±12)	55.2(±14)	71.8(±16)
10	Fisetin	50	55.5(±2)	60.2(±4)	50	10.6(±4)	30.4(±12)	
11	Rhamnetin	**			50	18.0(±11)	35.4(±7)	
20	Esculetin	50	69.4(±5)	54.8(±3)	**			
21	6,7-dihydroxy-4-methyl coumarin	50	46.6(±3)	32.8(±3)	**			

* *n* = 4. ** Compound not inhibitory with this enzyme.

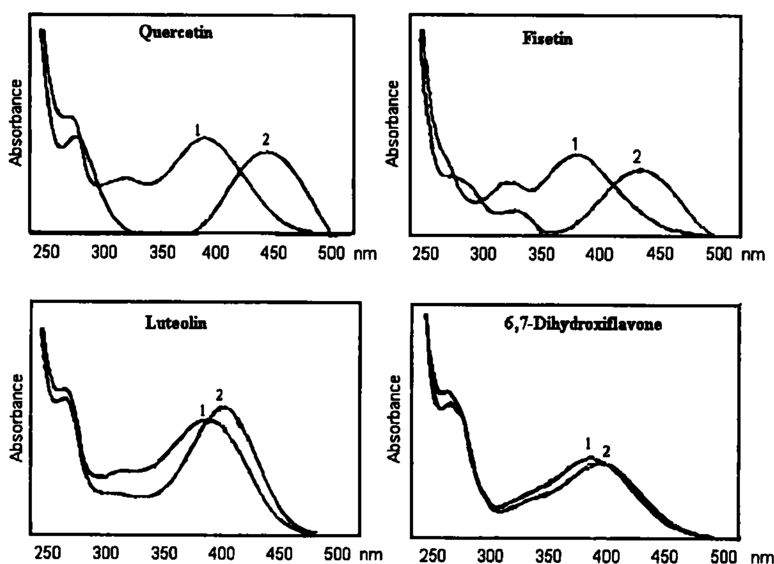

FIGURE 1 Recorded spectra of flavonoids in the absence (1) and presence (2) of added $ZnCl_2$ under the conditions of the enzymatic assays.

employed, the flavonoids were retained on the column, resolution of the substrate, internal standard, and hydrolysate was satisfactory, and the method thus fulfilled all the validation requirements for an enzymatic method.[20] Mean CP-A activity under the conditions described was 1.51 mM of hippuric acid min^{-1} (RSD 8.8%, $n = 16$). This is the first report, to our

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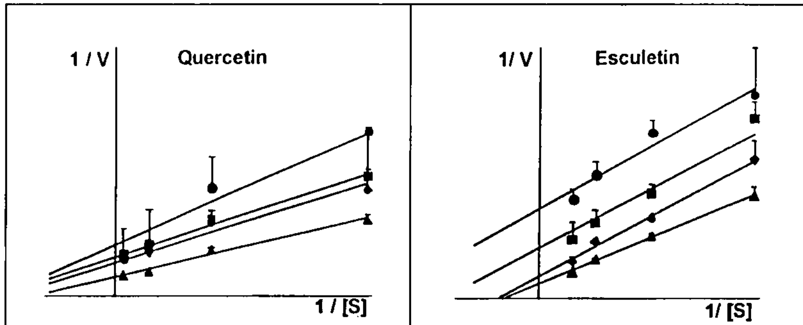

FIGURE 2 Lineweaver–Burk plots for LAP in presence of quercetin and esculetin. ▲ LAP control; ◆ quercetin 10 μM; ■ quercetin 25 μM; ● quercetin 60 μM. ▲ LAP control; ◆ esculetin 5 μM; ■ esculetin 10 μM; ● esculetin 50 μM.

TABLE III Effect of ultrafiltration on inhibitory activity

No.	*Compound*	*Conc.* (μM)	*LAP-inhibition* (%)†		*AP-M-inhibition* (%)†	
			Before ultrafiltration	*After ultrafiltration**	*Before ultrafiltration*	*After ultrafiltration**
6	7,8-dihydroxy flavone	40	29(±2)	—	**	
9	Quercetin	50	51(±9)	15(±3)[1] 16(±3)[2] 8(±5)[3]	22(±5)	25(±2)[1] 32(±3)[2] 35(±3)[3]

*Successive ultrafiltrations. **Compound not inhibitory with this enzyme. † $n = 4$. —: No inhibition.

TABLE IV Effect of flavonoids on the activity of carboxypeptidase A

No.	*Flavonoid*	*Conc.* (μM)	*Inhibition* (%)*
3	Luteolin	50	27(±5)
		150	61(±10)
4	3′,4′-dihydroxyflavone	150	—
8	Kaempferol	50	—
		150	23(±5)
9	Quercetin	50	25(±2)
		150	53(±4)
12	Morin	150	—
15	Eriodictyol	150	25(±3)
16	Taxifolin	150	10(±0.3)

* $n = 5$. —: No inhibition or less than 10%. Enzyme control: 1.51 (±0.13) $n = 16$.

knowledge, of CP-A activity based on a HPLC method and it is worth noting that the reproducibility obtained for this assay is very similar to that described for other enzyme assays by HPLC.[16] Table IV summarises the effects of flavonoid compounds on CP-A.

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DISCUSSION

The effect of the phenolic compounds tested on the metalloenzymes considered was dependent upon enzyme structure and conformation as well as upon the structure of the substance concerned. A particular structural feature of flavonoids that appeared to be conducive to interaction with the three metalloenzymes was the 2,3-double bond conjugating the A and B rings and conferring a planar structure which was virtually indispensable for inhibition of the three metallopeptidases. An appropriate hydroxylation profile for the flavonoid is a further essential prerequisite for inhibition of a given enzyme.

AP-M is a membrane-bound aminopeptidase containing one catalytic zinc ion per subunit.[7] It is inhibited by flavonoids with a 2,3-double bond and at least one free hydroxyl group on each aromatic ring (Table I, compounds **2**, **8**–**13**). The molar inhibitor to enzyme ratio was on the order of 10^5, and the effect was not reversed by ultrafiltration, a procedure that removes any free inhibitor or inhibitor released as a result of the dilution effect (Table III), indicating that the process of enzymatic inactivation was irreversible.[21] The rank order of potency of the flavonoids was: quercetin > fisetin ≈ morin ≈ myricetin ≈ kaempferol ≈ rhamnetin ≈ apigenin > luteolin, demonstrating that *o*-dihydroxylation was not indispensable and that 3-hydroxylation was conducive to interaction. The coumarins (**17**–**23**), with structures derived from benzo-α-pyrone, and the flavanol epicatechin (**1**), which lacks the carbonyl group at the 4-position and the double bond of γ-pyrone, were virtually ineffective, while the effect of the xanthone mangiferin (**24**) was similar to that of quercetin. These findings indicated that the carbonyl group at the 4-position was critical to activity, and only those substances with structures containing two aromatic rings either conjugated or fused with γ-pyrone were capable of reducing enzymatic activity.

LAP is a hexameric enzyme with two zinc ions per protomer. Its three-dimensional structure has been determined in complexes with different ligands formed by the enzymes.[3,22] Examination of the structure–activity relationships in the flavonoid series (**2**–**16**) (Table I) revealed stricter requirements for effective inhibition of LAP than of AP-M by the flavonoids. The results suggested certain general conclusions concerning common features of the inhibitors: (i) a planar structure of the chromone ring with sp^2 carbons at the 2 and 3 positions is required for significant inhibitory activity, (ii) *o*-dihydroxylation of one of the benzene rings is necessary for activity (compounds **3**, **4**, **6**, **9**, **10**). This requirement would be in accord with a catechol-type mechanism of inhibition, as it has been described for

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other enzymes,[23] the position of these OH groups on the rings being of major importance as seen by the higher potency of 3′,4′-dihydroxyflavone (**4**) as compared to 6,7-dihydroxy and 7,8-dihydroxyflavones (**5, 6**), (iii) the inhibitory effect can be reversed by ultrafiltration (Table III), and (iv) inhibition is non-competitive (see Figure 2), which means that the flavonoid does not interact with the active site of the enzyme. This view is also supported by non-recovery of enzymatic activity after addition of zinc salts (Table II).

It has to be stressed that when $ZnCl_2$ is added to the reaction medium the inhibition by active flavonols increases. The spectral data recorded (Figure 1) provide evidence for the instantaneous coordination of Zn with the carbonyl group at the 4-position and with the hydroxyl groups at the 3- and 5-positions, as demonstrated by the bathochromic shift of 50–70 nm in the initial band I of the UV spectra (quercetin, fisetin). Thus, electron delocalization favours semiquinone radical formation, facilitating the catechol-interaction with the protein and consequently explaining the increased inhibitory effect of these compounds. The non-competitive mechanism of inhibition also supports this conclusion.

The zinc coordination noted would be weaker in where any or all of the electron donor atoms at the 3- or 5-positions were missing. In support, compound **3** with no hydroxyl at the 3-position gives a lower coordination as is shown by a lower bathochromic shift (Figure 1) and consequently addition of Zn does not favour the inhibitory effect (Table II). Moreover, in the case of flavones that lack hydroxyl groups in the vicinity of the keto group (**4, 5**), namely, 3′,4′-dihydroxyflavone and 6,7-dihydroxyflavone, the effect of added zinc was very weak (Table II). Similar zinc effects were observed in the inhibitory activities of certain flavonoids on AP-M.

The ineffectiveness of rhamnetin (**11**) on LAP, an *o*-dihydroxylated flavonol at the 3′- and 4′-positions also bearing a methoxyl group at the 7-position, was due to the break in the dissociation sequence 7-OH ↔ 4′-OH ↔ 5-OH[24] caused by replacement of the hydroxyl group at the 7-position by the methoxyl group, preventing formation of the semiquinone radical on the B ring and blocking the catechol-like reactions. The occurrence of methylation (**7**) or glycosilation (**14**) also resulted in the complete loss of activity against LAP.

For inhibition of LAP by the coumarins (**17–23**), *o*-dihydroxylation of the benzo-α-pyrone ring was also necessary (**20**). The inhibitory action of esculetin was similar to that of active flavonoids ($IC_{50} = 8\,\mu M$), but due to its uncompetitive mechanism of inhibition (Figure 2), the planar structure (a requirement comparable to the planar structure required in the flavonoids) and the relatively small size of this molecule it was able to interact or

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gain access to sites on the enzyme affecting the conformation of the enzyme's active site, thereby altering both its affinity for the substrate, K_m, and the maximum reaction rate, V_{max}. The inhibitory effect was reversible and was likewise attributable to a catechol-type inhibition mechanism, much stronger for 6,7-dihydroxycoumarin (esculetin) (**20**).

The results for inhibition of CP-A by the flavonoids tested (Table IV) showed that only quercetin and luteolin, unsaturated at the 2- and 3-positions with an *o*-dihydroxylated B ring, had significant effects at 50 μM.

The present study has identified a novel form of biological action by flavonoids, namely, inhibition of zinc metallopeptidases that may contribute to antiinflammatory effects,[25] antiproliferative activity,[26] and chemopreventive action against carcinogenesis[27] by such compounds.

Acknowledgements

This study was supported by a grant from the University of Alcalá. (Project ref. 044/96.)

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