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Morelloflavone, a Novel Biflavonoid Inhibitor of Human Secretory Phospholipase A_2 With Anti-Inflammatory Activity

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ABSTRACT. The flavanonylflavone morelloflavone inhibited secretory phospholipase A_2 (PLA_2) *in vitro*, with a high potency on the human recombinant synovial and bee venom enzymes (IC_{50} = 0.9 and 0.6 μM, respectively). The inhibition was apparently irreversible. In contrast, the compound was inactive on cytosolic PLA_2 activity from human monocytes. Morelloflavone scavenged reactive oxygen species generated by human neutrophils (IC_{50} = 2.7 and 1.8 μM for luminol and lucigenin, respectively) but did not modify cellular responses such as degranulation or eicosanoid release. This biflavonoid exerted anti-inflammatory effects in animal models, with a potent inhibition of 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced ear inflammation in mice after topical administration. In this test, morelloflavone was found to decrease oedema and myeloperoxidase levels in ear homogenates (ID_{50} = 58.5 and 74.3 μg/ear, respectively). In contrast, this biflavonoid failed to modify arachidonic acid-induced ear inflammation or eicosanoid levels in ear homogenates. A significant anti-inflammatory effect was also observed in the mouse paw carrageenan edema after oral administration, with the highest inhibition at 3 hr after induction of inflammation. Morelloflavone is an inhibitor of secretory PLA_2 with selectivity for groups II and III enzymes and may be a pharmacological tool. In addition, it shows anti-inflammatory activity apparently not related to the synthesis of eicosanoids, but likely dependent on other mechanisms such as scavenging of reactive oxygen species. BIOCHEM PHARMACOL 53;5:733–740, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. biflavonoid; morelloflavone; phospholipase A_2; anti-inflammatory drugs; chemiluminescence; human neutrophil

The lipolytic enzyme PLA_2§ specifically releases arachidonic acid from the *sn*-2 position of phospholipids as a previous step for the synthesis of eicosanoids and platelet-activating factor. Most cells contain at least two forms of PLA_2: a 14 kDa secretory enzyme and an 85-kDa cytosolic PLA_2 [1, 2]. The secretory enzymes can be classified into group I and group II, based on their primary structures [3]. In addition, bee venom contains a PLA_2 included in group III [1].

Group II enzymes have been found at inflammatory sites in animal models, as well as in the synovial fluid of patients with rheumatoid arthritis and in various human inflammatory disease states, where a correlation exists between serum PLA_2 levels and disease activity [4]. Furthermore, the exogenous administration of secretory PLA_2s can induce or exacerbate inflammatory responses in animals [5–8]. Thus, group II secretory PLA_2s have been implicated in inflammatory processes, and an important effort has been made to develop pharmacological agents able to inhibit this enzyme activity and, presumably, to control inflammatory states.

A number of flavonoids possess anti-inflammatory activity [9, 10] and some of them can also act as weak PLA_2 inhibitors [11]. Recently, the potent inhibition of secretory PLA_2 by biflavones such as ochnaflavone and amentoflavone has been reported [12]. In the present study, we assessed the effects of the natural flavanonyl-flavone morelloflavone on PLA_2 activity, some human neutrophil functions, and experimental inflammation in mice.

MATERIALS AND METHODS

Reagents

Morelloflavone was isolated from *Garcinia spicata* (Clusiaceae) and identified by comparing its spectral data with those of authentic samples. The 5-lipoxygenase inhibitor ZM230,487, antibody against LTB_4, and human recombi-

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§ *Abbreviations*: TPA, 12-O-tetradecanoylphorbol 13-acetate; PLA_2, phospholipase A_2; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; PGE_2, prostaglandin E_2; FMLP, N-formyl-L-methionil-L-leucyl-L-phenylalanine; RAP, rat air pouch; PTK, palmityl trifluoromethyl ketone.

nant synovial PLA_2 were kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K. and scalaradial was a gift from Dr. S. De Rosa, Istituto per la Chimica di Molecole di Interesse Biologico, Arco Felice, Naples, Italy and bakuchiol from Prof. E. González, Universidad de Chile (Santiago, Chile). [9,10-^{3}H]oleic acid and L-3-phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] were purchased from Du Pont, (Itisa, Madrid, Spain); ([5,6,8,11,12,14,15(n)-^{3}H]PGE_2) and the LTC_4 radioimmunoassay kit were from Amersham Iberica, (Madrid, Spain). PTK was purchased from Universal Biologicals Ltd. (London, U.K.). The rest of the reagents were from Sigma Chem. (St. Louis, MO). *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Departamento de Microbiologia, Universidad de Valencia, Spain.

Assay of Secretory PLA_2

This activity was assayed using [^{3}H]oleate-labelled membranes of *Escherichia coli*, following a modification of the method of Franson et al. [13]. *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% (w/v) NaCl, and 0.6% (w/v) sodium dihydrogen orthophosphate, pH 5.0, and grown for 6–8 hr at 37°C in the presence of 5 μCi mL^{-1} [^{3}H]oleic acid (sp. act. 10 Ci $mmol^{-1}$) until growth approached the end of the logarithmic phase. After centrifugation at 2500 *g* for 10 min, the membranes were washed in buffer (0.7 M Tris-HCl, 10 mM $CaCl_2$, 0.1% bovine serum albumin, pH 8.0), resuspended in saline and autoclaved for 30–45 min. After washing and centrifugation, the membranes were frozen at −70°C. The phospholipid fraction incorporated at least 95% of the radioactivity. *Naja naja* venom enzyme (0.1 U), bee venom enzyme (0.005 U), human recombinant synovial enzyme (0.03 μg protein), or supernatants (10 μL) of exudates from 8-hr zymosan-injected RAP [14] was preincubated with test compound or vehicle (methanol) for 5 min at 37°C, in 250 μL of 100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5. After addition of 10 μL autoclaved oleate-labelled membranes, incubation proceeded for 15 min and was terminated in the presence of 100 μL ice-cold solution of 0.25% bovine serum albumin in 100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5 to a final concentration of 0.07% w/v. After centrifugation at 2500 *g* for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting. The inhibitory effect of test compounds was measured in relation to control enzyme activity in tubes containing enzyme and the inhibitor vehicle. For reversibility studies, the dilution method [15] and kinetic analysis of enzyme activity against enzyme concentration were used. In the first case, recombinant human synovial PLA_2 was preincubated for 10 min at 37°C with 5 μM morelloflavone. A 25-fold dilution of this mixture (0.2 μM morelloflavone) was then incubated for 15 min at 37°C with 10 μL labeled *E. coli* membranes. The reaction was terminated by addition of 100 μL ice-cold solution of 0.25% BSA and enzymatic activity was determined as above. The release of oleic acid as a function of bee venom PLA_2 concentrations was also determined in the absence and presence of morelloflavone. To test the influence of substrate on inhibitory activity, morelloflavone (5 μM) was incubated with bee venom PLA_2 (0.005 U) at different times in the presence of 5 μL or 50 μL of *E. coli* membranes.

Preparation of Human Neutrophils and Monocytes

Cells were isolated from the peripheral blood of healthy volunteers. The citrated blood was centrifuged at 200 *g* for 15 min at room temperature. The platelet-rich plasma was removed and the leukocytes contained in the residual blood were isolated by sedimentation with 2% (w/v) dextran in 0.9% NaCl at room temperature. The supernatant was centrifuged at 1200 *g* for 10 min at 4°C. Contaminating erythrocytes were lysed by hypotonic treatment. The pellet was resuspended in PBS, and Ficoll-hypaque was layered under the cell mixture. The cell gradient mixture was centrifuged at 400 *g* for 40 min at 20°C to obtain neutrophils and the monocyte and lymphocyte layer, which was removed and pelleted by centrifugation. The cell pellet was resuspended in RPMI-1640 media pH 7.4 with 10% fetal bovine serum and 2 mM L-glutamine and was incubated at a cell density of 10^7/mL in 60/15 mm tissue culture dishes. The cells were allowed to adhere for 2 hr at 37°C in a 5% CO_2 atmosphere incubator. The nonadherent cells were removed by vacuum suction of media followed by 2 washes with 1 mL of RPMI-1640. The adherent cells resulted in a greater than 90% pure monocyte population as assessed by differential staining.

Preparation of Human Cytosolic PLA_2

Cytosolic PLA_2 was prepared from human monocytes that were disrupted by sonication in 10 mM Hepes buffer pH 7.4, containing 0.32 M sucrose, 100 μM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride, and 100 μM leupeptin. The homogenated cells were centrifuged at 2000 *g* for 10 min at 4°C and the resulting supernatant further centrifuged at 100,000 *g* for 100 min at 4°C to obtain the cytosolic fraction.

Assay of Cytosolic PLA_2

1-Palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (57.0 mCi/mmol, 2×10^6 cpm) was dried under nitrogen, then suspended in 1 mL of 100 mM glycine buffer pH 9.0 containing 200 μM Triton X-100, 10 mM $CaCl_2$, 0.25 mg/mL BSA, and 40% v/v glycerol. The suspension was then sonicated to form mixed micelles of phospholipid and Triton X-100. The reaction was started by adding the enzyme (approximately 24 μg protein of cytosolic fraction from human monocytes) to a final volume of 100 μL of the assay mixture, which contained 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 150 mM NaCl, 40% glycerol, 1 mg/mL BSA, and 50 mM Hepes, pH 9.0. The substrate consisted of 5 μL

of micelles (10^4 cpm) containing dioleoyl glycerol at a molar ratio 2:1 [16]. Morelloflavone was dissolved in methanol and added to the reaction mixture just before the addition of the enzyme solution. The final concentration of methanol in the reaction mixture was less than 1%, showing no effect on enzyme activity. The reaction was stopped after a 60-min incubation period at 37°C by mixing with 0.5 mL of isopropyl alcohol:heptane:0.5 M H_2SO_4 (10:5:1). Heptane (0.7 mL) and water (0.2 mL) were then added, and the solution was vigorously mixed for 15 sec. The heptane phase was mixed with 100 mg silica gel 60 (Merck, 70–230 mesh) and centrifuged, and the radioactivity in each supernatant was measured [17].

Chemiluminescence

Neutrophils (2.5×10^6) were mixed with lucigenin (250 μM) or luminol (40 μM) in a total volume of 250 μL and stimulated with TPA (1 μM). The chemiluminescence was recorded in a Microbeta Trilux counter (Wallac, Turku, Finland) after 7 min, previously selected as the maximal production. Results are expressed as percentage inhibition of total luminiscence counts obtained with the generating system after subtracting the basal emission (cells without stimulus). Superoxide anions were also generated by the system hypoxanthine/xanthine oxidase [18]. The reaction mixture contained 50 mM KH_2PO_4-KOH pH 7.4, 1 mM EDTA, 100 μM hypoxanthine, and lucigenin (250 μM) or luminol (40 μM) in a total volume of 250 μL. The reaction was started by adding 0.2 mU or 10 mU of xanthine oxidase for luminol or lucigenin, respectively. Morelloflavone was added dissolved in 2.5 μL ethanol. Control incubations contained the same volume of ethanol. We had previously found that morelloflavone did not inhibit xanthine oxidase activity at the concentrations used following the formation of uric acid.

Elastase Release by Human Neutrophils

Neutrophils, 2.5×10^6 mL, were preincubated with test compound or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and FMLP (10 μM) for 10 min at 37°C. After centrifugation at 1200 *g* at 4°C, supernatants were incubated with N-tert-butoxy-carbonyl-L-alanine p-nitrophenyl ester (200 μM) for 10 min at 37°C [19]. The extent of p-nitrophenol release was measured at 414 nm in a microtiter plate reader.

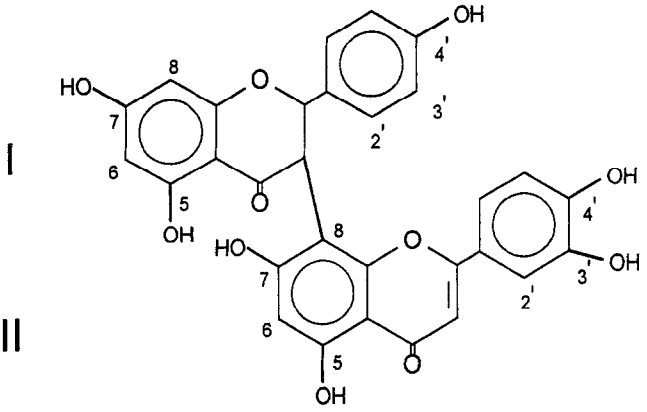

FIG. 1. Structure of morelloflavone.

Release of LTB_4 by Human Neutrophils

A suspension of human neutrophils (5×10^6/mL) in PBS was preincubated with test compounds or vehicle and then stimulated with 1 μM A23187 for 10 min at 37°C. After centrifugation at 1200 *g* for 10 min at 4°C, the supernatants were frozen at −80°C until the radioimmunoassay for LTB_4 was performed [20].

Mouse Ear Edema

Inflammation was induced by topical administration of TPA (2.5 μg) or arachidonic acid (2.0 mg) in 20 μL acetone to both surfaces of the right ear of Swiss mice (20–25 g). Test compounds dissolved in acetone were applied topically before TPA administration or 20 min before arachidonic acid administration. After 4 hr (TPA) or 1 hr (arachidonic acid), the animals were killed by cervical dislocation and equal sections of both ears were punched out and weighed [21]. The ear sections were homogenized and the PGE_2 and LTC_4 content in supernatants was determined by radioimmunoassay (arachidonic acid-induced edema), or myeloperoxidase activity was measured in supernatants (TPA-induced edema). PGE_2 radioimmunoassay was performed as indicated previously [20] and LTC_4 was assayed using a radioimmunoassay kit from Amersham Iberica (Madrid, Spain).

Myeloperoxidase Assay

The reaction mixture contained 2.5 μL of ear homogenate supernatant, 190 μL PBS, 20 μL 0.22 M NaH_2PO_4 (pH 5.4), 20 μL 0.026% (v/v) H_2O_2, and 20 μL 18 mM tetra-

TABLE 1. Effect of morelloflavone on secretory PLA_2 activity

	Naja naja venom	Bee venom	Human recombinant	RAP + zymosan
Morelloflavone	42.5 ± 3.8%†	0.6 (0.3–1.0) μM	0.9 (0.8–1.0) μM	18.9 (10.8–22.3) μM
Mepacrine	0.2 (0.1–0.3) mM	0.6 (0.4–0.8) mM	48.9 ± 2.6%†	53.2 ± 1.8%†
Bromophenacyl bromide	0.3 (0.2–0.5) mM	4.8 (4.2–5.8) mM	1.2 (0.7–1.8) mM	26.2 ± 2.7%*

Values are IC_{50} with 95% confidence limits calculated from at least 4 statistically significant points (each of them the mean of 6 experimental values) or means ± SEM ($n = 6$) of percentages of inhibition at the concentration of 1 mM. *$P < 0.05$; †$P < 0.01$.

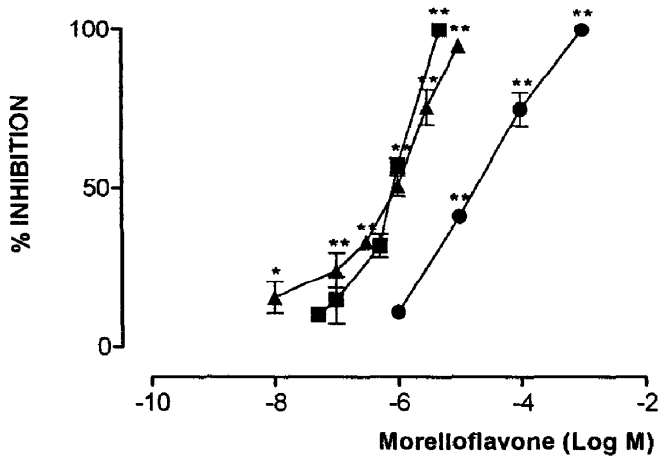

FIG. 2. Dose-response curve showing the effect of morelloflavone on secretory PLA_2 activity. Data are means ± SEM (n = 6). *P < 0.05, **P < 0.01. (■) Human recombinant synovial PLA_2; (▲) bee venom PLA_2; (●) RAP + zymosan PLA_2. Enzyme was preincubated with morelloflavone for 5 min at 37°C and, after addition of oleate-labelled membranes, incubation proceeded for 15 min. The inhibitory effect of morelloflavone was measured in relation to control enzyme activity in tubes containing enzyme and the inhibitor vehicle (methanol).

methylbenzidine in 8% (v/v) aqueous dimethylformamide. After a 10-min reaction at 37°C, 30 μL 1.46 M sodium acetate, pH 3.0, was added and absorbance at 620 nm was read using a Multiskan MCC/340 microtiter plate reader (Labsystems, Helsinki, Finland) [22, 23].

Mouse Paw Edema

Edema was induced by a modification of the technique of Sugishita et al. [24]. Female Swiss mice (20–25 g) were fasted for 12 hr with free access to water. Drugs or vehicle (ethanol, Tween 80, distilled water: 5/5/90, v/v/v) was administered orally (0.5 mL) 1 hr before injection of carrageenan (0.05 mL; 3% w/v in saline) into the subplantar area of the right hind paw, and the volumes of injected and contralateral paws were measured at 1, 3, and 5 hr after induction of edema by using a plethysmometer (Ugo Basile).

Data Analysis

Data were expressed as means ± SEM. Statistical analysis was performed by 1-way analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons.

TABLE 2. Effect of morelloflavone on cytosolic PLA_2 activity

	% Inhibition (100 μM)	IC_{50}
Morelloflavone	4.5 ± 2.9	N.D.
PTK	94.7 ± 1.7*	0.3 (0.1–0.4) μM

PTK, palmityl trifluoromethyl ketone; N.D., not determined. Data values represent means ± SEM of percentages of inhibition (n = 6) and IC_{50} (μM) with 95% confidence limits. *P < 0.01.

TABLE 3. Effect of morelloflavone on human recombinant synovial PLA_2 activity before and after dilution

	% Inhibition	
	Before dilution	After 25-fold dilution
Morelloflavone (5 μM)	88.0 ± 0.8*	93.1 ± 2.8*
Scalaradial (3 μM)	98.7 ± 2.8*	98.0 ± 1.3*

Values are means ± SEM (n = 6). *P < 0.01.

RESULTS

The effects of morelloflavone (Fig. 1) on several secretory PLA_2 activities were examined (Table 1). A concentration-dependent inhibitory effect was observed for the human recombinant synovial enzyme as well as for the bee venom enzyme (Fig. 2). Recently, we reported the presence of a PLA_2 activity that does not show selectivity for arachidonoyl phospholipids in the RAP injected with zymosan [14]. This activity was also inhibited by morelloflavone in a concentration-dependent manner. This flavonoid was a potent inhibitor of the first two enzymes, with IC_{50} values under the μM range and a lower potency of RAP + zymosan activity. In contrast, morelloflavone weakly inhibited the type I secretory PLA_2 present in *Naja naja* venom and did not affect cytosolic PLA_2 (Table 2), which was potently inhibited by the selective inhibition PTK. We also investigated the type of inhibition exerted by morelloflavone on secretory PLA_2. The irreversibility of inhibition was assessed by the dilution method (Table 3). A potent irreversible inhibitor of secretory PLA_2 (scalaradial) was also tested as reference in this system. Scalaradial inhibited this enzyme activity before and after dilution in a similar manner. Morelloflavone (at the final concentration of 5 μM) in-

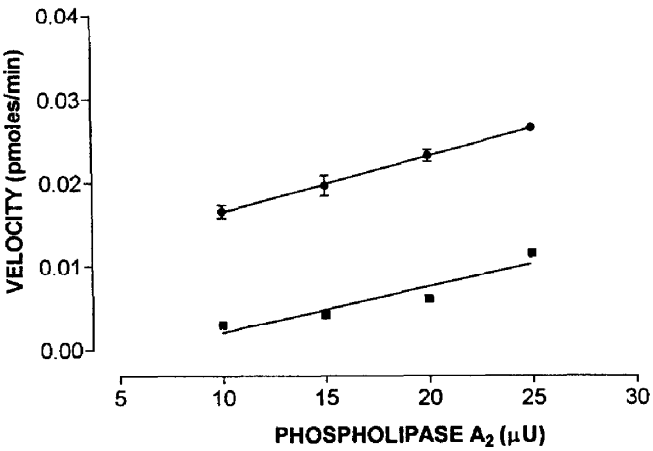

FIG. 3. Activity of bee venom PLA_2 as a function of enzyme concentration in the absence and presence of morelloflavone. (●) Control; (■) 1 μM morelloflavone. Data are the means ± SEM (n = 6). Different enzyme concentrations were preincubated with vehicle (control) or morelloflavone (1 μM) for 5 min at 37°C and, after addition of substrate, incubation proceeded for 15 min.

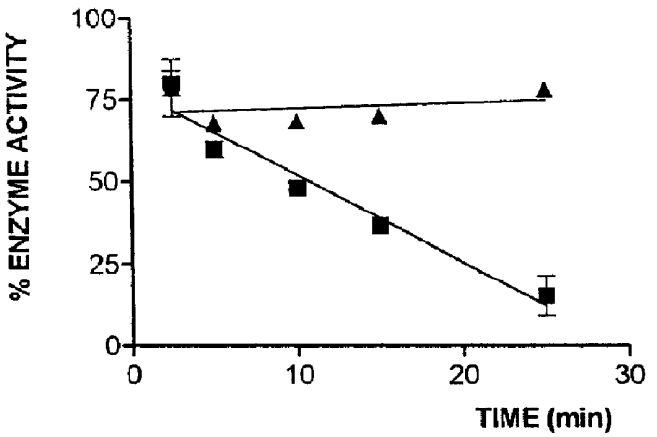

FIG. 4. Influence of substrate concentration on the inhibitory effect of morelloflavone on bee venom PLA_2. Enzyme was incubated with morelloflavone for different times in the presence and absence of an excess of substrate. (■) 5 μM morelloflavone + 5 μL *E. coli* membranes (3.5 μM phospholipid substrate); (▲) 5 μM morelloflavone + 50 μL *E. coli* membranes (35 μM phospholipid substrate). Data are the means ± SEM (n = 3).

hibited human recombinant synovial PLA_2 by 88% and, after a 25-fold dilution (final concentration 0.2 μM), the observed inhibition was even higher, suggesting an apparent irreversible inhibition of human recombinant synovial PLA_2.

The kinetic analysis of enzyme activity as a function of enzyme concentration in the absence and presence of morelloflavone also indicated an irreversible inhibition of bee venom PLA_2 by morelloflavone (Fig. 3) because there was no significant difference in the slopes of the straight lines [25].

The inactivation of bee venom PLA_2 by morelloflavone in the presence of 3.2 μM substrate was dependent on the time of incubation (Fig. 4). When the substrate was added to the enzyme + inhibitor mixture at a 10-fold higher concentration, the inhibition by morelloflavone was markedly decreased and independent of time, suggesting competition with the substrate for enzyme binding.

The influence of morelloflavone on some cellular functions relevant to the inflammatory process was studied using human neutrophils. This flavonoid potently inhibited chemiluminescence (Table 4) produced by human neutrophils stimulated by TPA, and also by the system hypoxanthine/xanthine oxidase, whereas it did not affect degranulation (measured as elastase release) or LTB_4 synthesis and release (Table 5), responses inhibited by the reference compounds bakuchiol or ZM230,487, respectively.

TABLE 5. Effect of morelloflavone on elastase release and LTB_4 synthesis and release by human neutrophils

	% Inhibition	
	Elastase release	**LTB_4 synthesis**
Morelloflavone (10 μM)	10.0 ± 2.1	13.9 ± 4.7
Bakuchiol (10 μM)	37.3 ± 4.1*	1.7 ± 1.7
ZM230,487 (10 μM)	N.D.	100 ± 0.0*

Values are means ± SEM (n = 6); *P < 0.01; N.D., not determined.

Anti-inflammatory effects of morelloflavone were observed after topical or oral administration. This flavonoid inhibited the edema induced by topical application of TPA dose-dependently, with a potency higher than that of indomethacin (Fig. 5). The calculated ID_{50} and 95% confidence limit values were 58.5 (48.3–59.2) and 191.6 (141.9–215.4) μg/ear for morelloflavone and indomethacin, respectively. As seen in Fig. 6, both compounds affected myeloperoxidase levels in a similar way, with ID_{50} values of 74.3 (53.1–89.0) and 74.6 (30.7–119.4) μg/ear, respectively. In contrast, morelloflavone failed to modify arachidonic acid-induced ear edema or eicosanoid levels (Table 6). As expected, indomethacin nearly abolished PGE_2 levels in this experimental model and inhibited edema at the highest dose tested.

After oral administration, morelloflavone dose dependently reduced carrageenan-induced edema in mice 3 hr and 5 hr after induction of inflammation (Fig. 7). The dose of 150 mg/kg also exerted significant inhibitory effects on swelling at the 1-hr determination. The highest effect was observed at the 3-hr determination, with percentages of inhibition of 24.8 ± 1.0 (P < 0.01, n = 12), 41.6 ± 1.9 (P < 0.01, n = 12) and 51.3 ± 2.8 (P < 0.01, n = 12) for the doses of 50, 100, and 150 mg/kg, respectively. In this test, indomethacin administered p.o. at 10 mg/kg inhibited paw edema by 22.4 ± 2.9% (P > 0.05, n = 6), 46.2 ± 5.2% (P < 0.01, n = 6) and 53.1 ± 7.1% (P < 0.01, n = 6) at 1, 3, and 5 hr after carrageenan administration, respectively.

DISCUSSION

PLA_2 of different types have been implicated in the hydrolysis of *sn*-2-arachidonate, although cytosolic PLA_2 seems to be the main agent responsible for receptor-coupled ara-

TABLE 4. Effect of morelloflavone on chemiluminescence

	Human neutrophil		Hypoxanthine/xanthine oxidase	
	Luminol	**Lucigenin**	**Luminol**	**Lucigenin**
Morelloflavone	2.7 (1.6–4.5) μM	1.8 (0.8–2.9) μM	2.2 (1.1–4.1) nM	46.6 (31.6–161.0) nM
Fraxetin	1.0 (0.7–1.8) μM	99.7 (39.2–339.8) nM	3.1 (2.5–4.0) nM	5.5 (4.1–6.7) nM

Values are IC_{50} with 95% confidence limits calculated from at least 4 statistically significant points (each of them the mean of 6 experimental values).

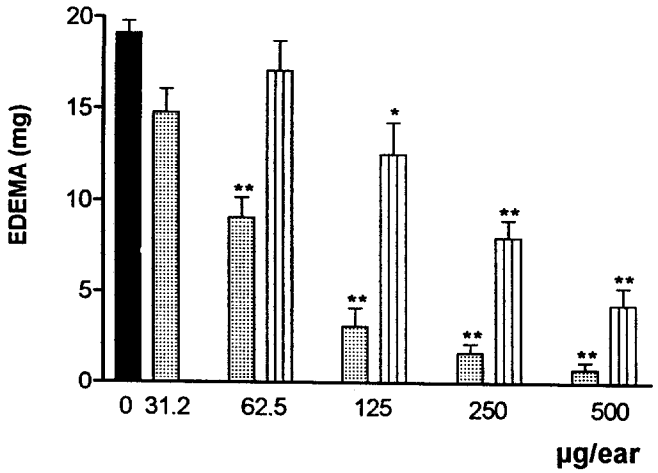

FIG. 5. Effect of morelloflavone on ear edema induced by TPA in mice. Data are means ± SEM (n = 6). *P < 0.05, **P < 0.01; (■) control; (▨) morelloflavone; (▥) indomethacin. Drugs were topically administered at the time of TPA application. After 4 hr, animals were killed and ear sections were punched out and weighed to measure the edema.

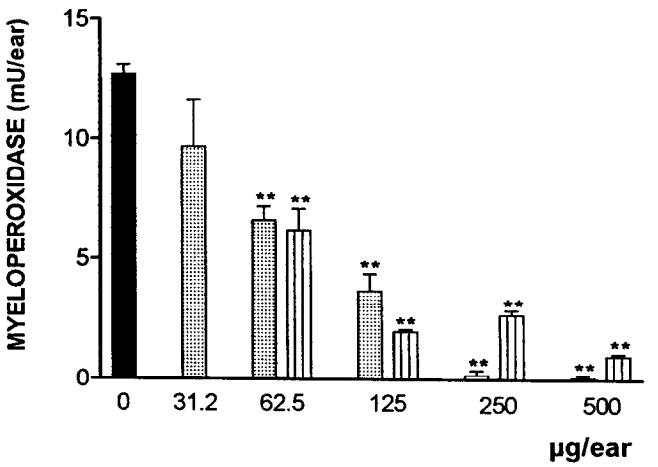

FIG. 6. Effect of morelloflavone on myeloperoxidase activity of ear homogenates in TPA-induced edema in mice. Data are means ± SEM (n = 6). **P < 0.01. (■) control; (▨) morelloflavone; (▥) indomethacin. Drugs were topically administered at the time of TPA application. After 4 hr, animals were killed and ear sections were punched out and homogenized to measure myeloperoxidase levels, as indicated in Methods.

chidonic acid release [26]. On the other hand, cytokine-induced synthesis and secretion of group II PLA_2 could contribute to PGE_2 synthesis in some cell types [27, 28]. It is likely that distinct PLA_2 activities can mobilize different pools of arachidonic acid for prostanoid generation [29]. Secretory PLA_2 could also participate in the regulation of cytotoxic proteins and superoxide anion release by human eosinophils [30], and its presence in inflammatory exudates could contribute to the degradation of bacterial phospholipids [31].

Morelloflavone is an inhibitor of secretory PLA_2 *in vitro* because it potently inhibited human synovial and bee venom PLA_2 with IC_{50} values of 0.9 and 0.6 µM, respectively. In addition, it showed a lower potency on the RAP + zymosan enzyme and a weak effect on the *Naja naja* venom enzyme, whereas this biflavonoid did not show any effect on cytosolic PLA_2. On the other hand, morelloflavone did not modify the release of LTB_4 in human neutrophils, which could depend on the lack of activity on cytosolic PLA_2.

The inhibition of secretory PLA_2 activity has been reported for some flavonoids, although they exhibited a low potency as inhibitors [11, 32]. Biflavone dimers of apigenin such as ochnaflavone (I-3′-O-II-4′ bond) and amentoflavone (I-3′, II-8 bond) have shown potent inhibitory effects on rat platelet PLA_2 with IC_{50} values of *ca.* 3 µM and irreversible inhibition [12]. Our results with morelloflavone confirm the ability of biflavonoids as inhibitors of group II PLA_2. This compound possesses free hydroxyl groups at I-5, II-5′, and II-7, which have been related to PLA_2 inhibition in biflavonoids [12], and exhibits a potency higher than that of other biflavonoids.

The inhibition of human recombinant PLA_2 was apparently irreversible, although our results also suggest that morelloflavone could compete with the substrate for a site on the enzyme. A possible interaction of morelloflavone with phospholipids may not be excluded, because it has been demonstrated that flavonoid monomers like quercetin form a complex enzyme inhibitor and also interact with the substrate [11].

Morelloflavone has shown anti-inflammatory activity in mice with potent inhibitory effects on TPA-induced edema (ID_{50} = 58.5 µg/ear) and the myeloperoxidase content of inflamed ears, an index for neutrophil migration. TPA specifically activates protein kinase C, resulting in activation

TABLE 6. Effect of morelloflavone on arachidonic acid-induced mouse ear edema

	Edema (mg)	PGE_2 (ng/ear)	LTC_4 (ng/ear)
Control	11.4 ± 1.1	120.6 ± 17.4	23.4 ± 2.8
Morelloflavone (0.5 mg/ear)	12.2 ± 1.6	155.7 ± 14.5	23.7 ± 1.8
(1.5 mg/ear)	13.0 ± 0.8	111.8 ± 6.9	21.4 ± 1.9
Indomethacin (0.5 mg/ear)	8.0 ± 1.1	13.3 ± 2.5*	22.4 ± 1.4
(1.5 mg/ear)	5.3 ± 1.0*	2.9 ± 0.5*	19.0 ± 2.1

Values are means ± SEM (n = 6–12); *P < 0.01. Ear sections were homogenized and PGE_2 and LTC_4 levels were determined by radioimmunoassay.

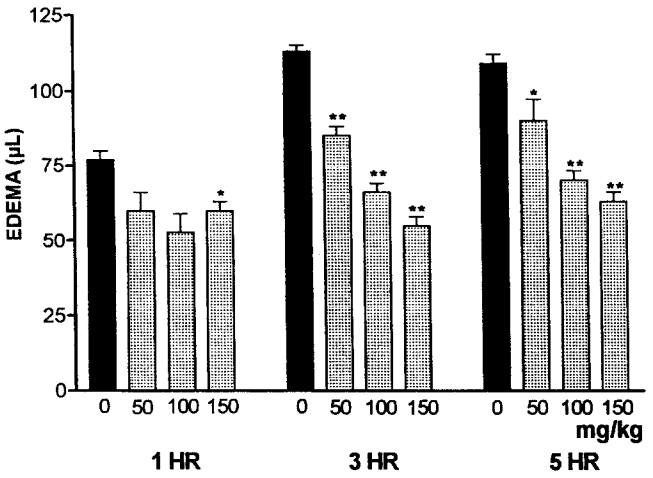

FIG. 7. Effect of morelloflavone on carrageenan mouse paw edema, 1, 3, and 5 hr after the induction of inflammation. (■) control; (▨) morelloflavone. Data are means ± SEM (n = 6–12). *$P < 0.05$; **$P < 0.01$. Morelloflavone or indomethacin was administered orally 1 hr before carrageenan injection.

of phospholipase A_2 [33] and TPA-induced edema could be related to prostaglandin and leukotriene formation [34], although other mediators, such as platelet-activating factor, could also participate [35]. On the contrary, morelloflavone did not inhibit arachidonic acid-induced edema nor affect arachidonic acid metabolism by cyclo-oxygenase or 5-lipoxygenase activities, because topical application of morelloflavone did not modify PGE_2 or LTC_4 levels in homogenates of ears treated with arachidonic acid.

Some inhibitors of group II PLA_2 are able to decrease the inflammatory response to carrageenan in the rat [36, 37]. In the model we have used, the carrageenan mouse paw edema, the second phase of this response (2–5 hr), is dependent on prostaglandin synthesis [24] and was inhibited by morelloflavone with a potency lower than that of the selective cyclo-oxygenase inhibitor indomethacin.

A number of inhibitors of secretory PLA_2 activity recently described [12, 37, 38] are valuable experimental tools and some of them affect inflammatory responses. Our results indicate that morelloflavone is an inhibitor of secretory PLA_2, with selectivity for group II and III enzymes, and may be a pharmacological tool to establish the pathophysiological roles of PLA_2 enzymes. On the other hand, we have shown the anti-inflammatory activity of this flavonoid after topical or oral administration, one apparently not related to the synthesis of eicosanoids. These studies suggest other mechanisms by which morelloflavone might mediate its anti-inflammatory activity, because this flavonoid is a potent scavenger of reactive oxygen species like superoxide, which play an important role in the production of tissue damage during inflammation [39].

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