Synthesis and Pharmacological Evaluation of 2′-Hydroxychalcones and Flavones as Inhibitors of Inflammatory Mediators Generation

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2′-Hydroxy-3,4-dimethoxy-3′,4′-dimethylchalcone (**3a**), 2′-hydroxy-3′,4′,3,4-tetramethoxychalcone (**3b**), and their corresponding flavones, 3′,4′-dimethoxy-7,8-dimethylflavone (**4a**) and 3′,4′,7,8-tetramethoxyflavone (**4b**), were prepared from 3,4-dimethoxycinnamic acid and the respective phenol. The four compounds inhibited enzymic lipid peroxidation and showed weak peroxyl scavenging activity. They also reduced LTB_4 release from human neutrophils stimulated by A23187. The chalcone **3b** was the only compound able to inhibit in a concentration-dependent way, synovial human recombinant phospholipase A_2 activity, human platelet TXB_2 generation, and human neutrophil degranulation. This chalcone exerted topical antiinflammatory effects in mice.

Introduction

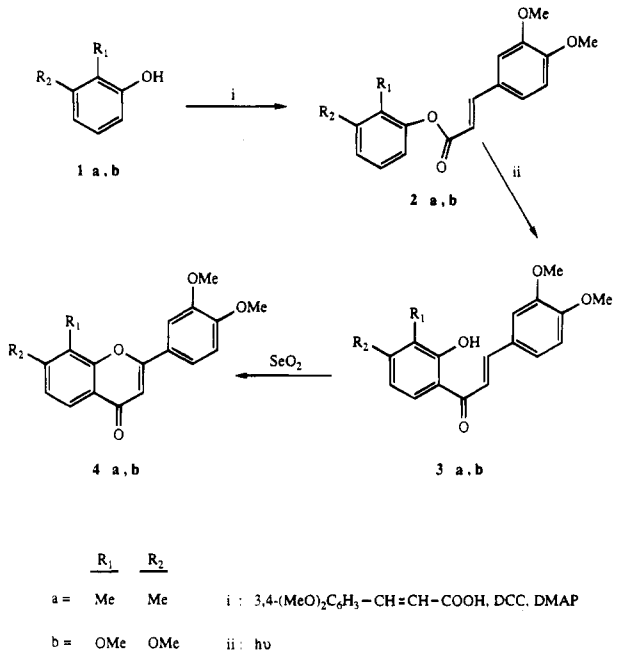
There is growing interest in the pharmacological potential of natural products. In recent years, we have focused on the study of flavonoids as inhibitors of oxidative reactions[1-5] and as antiinflammatory agents.[6-10] Most of the flavonoids studied thus far are polyhydroxylated derivatives which appear capable of selectively reacting with free radicals or systems related to the induction of inflammatory processes. Nevertheless, very few data are available on the influence of lipophilic substituents on the antioxidant or antiinflammatory activity of this class of natural products. In this study, we synthesized four new flavonoid derivatives, two chalcones and their corresponding flavones, possessing methyl and methoxyl substituents at positions related to inhibition of the 5-lipoxygenase pathway, like C-7, C-8 and C-3′, C-4′ in polyhydroxylated flavones,[8] to examine their effects on the generation of inflammatory mediators as well as on some free radical-mediated reactions.

Chemistry

The route followed for the preparation of 2′-hydroxychalcones **3a,b** and flavones **4a,b** is illustrated in Scheme 1. In the first step, 3,4-dimethoxycinnamic acid was condensed with the respective phenols **1** by means of *N,N′*-dicyclohexylcarbodiimide[11-14] using 4-(*N,N*-dimethylamino)pyridine as catalyst. The second step was the photo-Fries rearrangement of the resulting cinnamates **2**, which afforded the corresponding 2′-hydroxychalcones **3a,b**. Related processes have been reported in the literature[15-19] as an efficient alternative entry to the synthesis of flavonoids. The chalcones **3** were purified by column chromatography and identified by IR, ^{1}H-NMR, and MS spectrometry.

The last step was the oxidative cyclization of the 2′-hydroxychalcones **3** to flavones **4** using selenium dioxide as oxidant and dimethyl sulfoxide as solvent.[20]

Scheme 1



Biological Results and Discussion

Antioxidant or free radical scavenging activity of flavonoids has been related to the number and position of free hydroxyl groups, which could act by their hydrogen donating capability.[2,3,21,22] As expected, the flavonoids tested were inactive on nonenzymic lipid peroxidation (Table 1) and unable to interact with superoxide or hydroxyl radicals (data not shown). Nevertheless, they showed a weak peroxyl scavenging activity and inhibited lipid peroxidation induced by CCl_4/NADPH or Fe^{3+}-ADP/NADPH, probably by interfering with microsomal enzymic systems. As seen in Table 1, flavones are more active than chalcones in the last assay.

Inhibition of phospholipase A_2 (PLA_2) activity offers an approach for the treatment of inflammatory and allergic disorders. Secretory PLA_2s are released upon cell activation and have attracted attention because of their ability to cleave the *sn*-2-acyl bond of phospho-

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Table 1. Effect of Chalcones and Flavones on Microsomal Liquid Peroxidation Induced by Different Systems and Peroxyl Scavenging[a]

	Fe^{2+}/ascorbate		CCl_4/NADPH		Fe^{3+}-ADP/NADPH		peroxyl scavenging	
	%*I*(100 μM)	IC_{50} (μM)	%*I*(100 μM)	IC_{50} (μM)	%*I*(100 μM)	IC_{50} (μM)	%*I*(100 μM)	IC_{50} (μM)
3a	8.2 ± 0.0		55.3 ± 1.5**	95.0	51.0 ± 11.0**		28.8 ± 3.7**	
3b	0		57.2 ± 1.9**	68.6	33.1 ± 2.2**		40.0 ± 2.2**	
4a	0		26.2 ± 2.0**		89.6 ± 3.0**	29.6	37.4 ± 1.2**	
4b	0		58.2 ± 0.1**	56.6	93.2 ± 1.4**	62.6	30.0 ± 3.3**	
propyl gallate	100**	4.9	100**	4.5	100**	8.0	100**	33.9

[a] Inhibition percentages (%*I*) at 100 μM are expressed as means ± SEM. **$p < 0.01$, $n = 6$.

Table 2. Effect of Chalcones and Flavones on Synovial Human Recombinant Phospholipase A_2, TXB_2 Generation, and Elastase Release

	% inhibition		
	hr-PLA_2	TXB_2 generation	elastase release
3a (100 μM)	27.5 ± 2.9*	28.1 ± 9.4*	39.6 ± 3.1**
3b (100 μM)	55.0 ± 3.5**	77.2 ± 3.5**	87.6 ± 2.4**
4a (100 μM)	25.7 ± 1.8*	37.4 ± 3.5**	9.6 ± 3.3
4b (100 μM)	46.2 ± 8.8**	38.6 ± 5.9*	41.7 ± 5.3**
mepacrine (1 mM)	48.9 ± 2.6**		
indomethacin (100 μM)		100**	31.9 ± 4.9*

[a] Inhibition percentages are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$, $n = 6$.

glycerides to generate the precursor of eicosanoids, arachidonic acid.[23] The inhibition of synovial human recombinant PLA_2 (hr-PLA_2) is shown in Table 2. Enzyme activity is inhibited by **3b** in a concentration-dependent fashion with an inhibitory concentration 50% (IC_{50}) of 40.2 μM. This chalcone is more potent than the reference inhibitor mepacrine, and besides, it showed selectivity for this PLA_2 activity (group II of secretory enzymes) since **3b** did not exert any effect on either *Naja naja* or bee venom PLA_2.

In human polymorphonuclear leukocytes (PMNs) no release of cytoplasmic lactate dehydrogenase was observed, thus excluding a cytotoxic effect of flavonoids at the concentrations used. The four compounds failed to modify the rate of superoxide generation (data not shown), but they were particularly efficient in inhibiting the 5-lipoxygenase-derived mediator, LTB_4 (Figure 1), whereas they showed a lower inhibitory effect on TXB_2 generation (Table 2). This selectivity for the lipoxygenase pathway has been previously reported previously for 7,8,3′,4′-hydroxyflavone derivatives.[8] Chalcone **3b** concentration-dependently decreased the levels of both eicosanoids (IC_{50} = 18.3 and 33.7 μM, respectively) and was also effective in inhibiting elastase release (Table 2) with an IC_{50} of 9.9 μM. Since the phospholipid-splitting activity is less sensitive to inhibition by **3a**, **4a**, and **4b**, it is likely that the primary site of action for these three compounds is at the level of the 5-lipoxygenase pathway (IC_{50} = 24.4, 7.6, and 10.4 μM, respectively, for inhibition of LTB_4 generation) rather than the mobilization of arachidonate from membrane phospholipids.

Furthermore, the *in vitro* antiinflammatory profile of **3b** was confirmed *in vivo* after topical administration (Figure 2) since it inhibited the mouse ear edema induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), with a potency similar to that of indomethacin (IC_{50} = 146.2 and 168.0 μg/ear, respectively). On the other hand, it would seem possible that the decrease in eicosanoid generation caused by **3b** results in part from

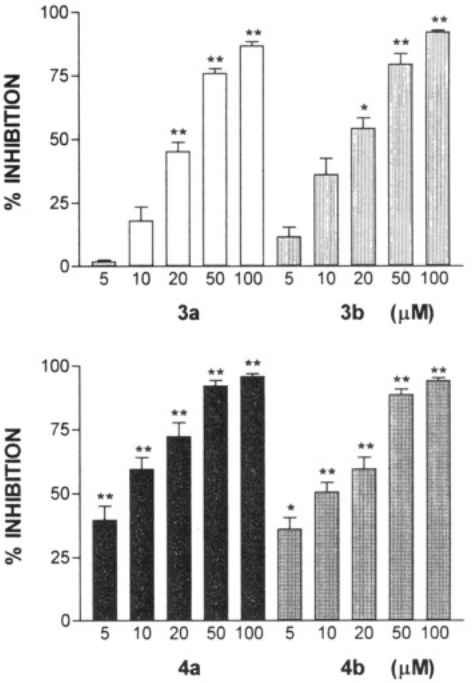

Figure 1. Effect of chalcones and flavones on LTB_4 generation in human PMNs. Results show percentages of inhibition (means ± SEM) for $n = 6$. *$P < 0.05$, **$p < 0.01$.

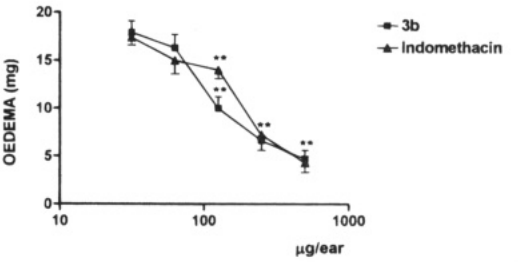

Figure 2. Effect of chalcone **3b** and indomethacin on mouse ear oedema induced by TPA. Results show milligrams of oedema (means ± SEM) for $n = 6$. **$p < 0.01$.

inhibition of PLA_2 and may contribute to the antiinflammatory activity of this flavonoid.

Experimental Section

Chemistry. The IR spectra were obtained in carbon tetrachloride solutions or potassium bromide pellets with a Perkin-Elmer 843 spectrometer; absorptions (cm^{-1}) are given only for the carbonyl bands. ^{1}H-NMR spectra were recorded on a 300 MHz Varian (Unity 300) instrument in $CDCl_3$ solutions; chemical shifts are reported as δ values (ppm) using Me_4Si as internal standard. Mass spectra were determined on a VG Analitica Fisons spectrometer; the *m/z* ratios and the relative intensities are indicated for the significant peaks.

Isolation and purification were done by column chromatography on silica gel Merck 60, 70−230 mesh.

General Procedure for the Preparation of 2′-Hydroxychalcones (3a,b). *N,N′*-Dicyclohexylcarbodiimide (0.98 g, 4.8

mmol) in dichloromethane (10 mL) was added dropwise to a solution of 3,4-dimethoxycinnamic acid (1.00 g, 4.8 mmol), the corresponding phenol (4.8 mmol), and 4-(*N*,*N*-dimethylamino)-pyridine (0.05 g) in dichloromethane. The mixture was stirred for 15 min at room temperature, then filtered to remove the precipitated *N*,*N*'-dicyclohexylurea, concentrated under reduced pressure, and purified by column chromatography, using dichloromethane as eluent. Solutions of the resulting esters in dichloromethane (300 mL) were placed in a quartz immersion well photoreactor with a 125-W medium-pressure mercury lamp and irradiated for 10 h under magnetic stirring. After this time, the photolysis mixture was vacuum-concentrated and the residue submitted to column chromatography using a 1:4 mixture of dichloromethane–hexane. Chalcones **3a** and **3b** were obtained in 12 and 15% yield, respectively.

Oxidation of 2'-Hydroxychalcones 3a,b to Flavones 4a,b. This oxidation was accomplished by treating a solution of the 2'-hydroxychalcone (0.25 g) in dimethyl sulfoxide (7 mL) with selenium dioxide (0.22 g) at 120 °C during 1 h. Then the selenium dioxide was filtered, and cold water was added. The resulting yellow solid was filtered and purified by column chromatography using a 9:6:1 mixture of dichloromethane–ethyl acetate–ethanol as eluent. This afforded the expected flavones **4a** (65%) and **4b** (61%).

2'-Hydroxy-3,4-dimethoxy-3',4'-dimethylchalcone (3a): IR 1620 cm^{-1} (C=O); ^1H NMR δ 13.40 (s, 1 H, OH), 7.86 (d, J = 15 Hz, 1 H, C*H*=CHCO), 7.68 (d, J = 9 Hz, 1 H, 6'-ArH), 7.51 (d, J = 15 Hz, 1 H, CH=C*H*CO), 7.25 (dd, J_a = 9 Hz, J_b = 2 Hz, 1H, 6-ArH), 7.18 (d, J = 2 Hz, 1 H, 2-ArH), 6.90 (d, J = 9 Hz, 1 H, 5-ArH), 6.74 (d, J = 9 Hz, 1 H, 5'-ArH), 3.95 (br s, 6H, 2 OCH_3), 2.33 (s, 3 H, CH_3), 2.21 (s, 3 H, CH_3); MS *m/z* 312 (84), 297 (78), 191 (100), 164 (46) 151 (17); HRMS (EI 70 eV) calcd for $C_{19}H_{20}O_4$ *m/z* 312.1362 (M^+), found 312.1365.

2'-Hydroxy-3',4',3,4-tetramethoxychalcone (3b): IR 1620 cm^{-1} (C=O); ^1H NMR δ 13.13 (s, 1 H, OH), 7.86 (d, J = 15 Hz, 1 H, C*H*=CHCO), 7.70 (d, J = 9 Hz, 1 H, 6'-ArH), 7.44 (d, J = 15 Hz, 1 H, CH=C*H*CO), 7.25 (dd, J_a = 9 Hz, J_b = 2 Hz, 1H, 6-ArH), 7.16 (d, J = 2 Hz, 1 H, 2-ArH), 6.91 (d, J = 9 Hz, 1 H, 5-ArH), 6.54 (d, J = 9 Hz, 1 H, 5'-ArH), 3.97 (s, 3 H, OCH_3), 3.96 (s, 3 H, OCH_3), 3.94 (s, 3 H, OCH_3), 3.92 (s, 3 H, OCH_3); MS *m/z* 344 (100), 191 (9), 180 (17), 164 (22); HRMS (EI 70 eV) calcd for $C_{19}H_{20}O_6$ *m/z* 344.1260 (M^+), found 344.1273.

3',4'-Dimethoxy-7,8-dimethylflavone (4a): IR 1630 cm^{-1} (C=O); ^1H NMR δ 7.98 (d, J = 9 Hz, 1 H, 5-ArH), 7.58 (dd, J_a = 9 Hz, J_b = 2 Hz, 1 H, 6'-ArH), 7.41 (d, J = 2 Hz, 1 H, 2'-ArH), 7.22 (d, J = 9 Hz, 1 H, 6-ArH), 7.00 (d, J = 9 Hz, 1 H, 5'-ArH), 6.74 (s, 1 H, COCH), 3.98 (s, 3 H, OCH_3), 3.97 (s, 3 H, OCH_3), 2.52 (s, 3 H, CH_3), 2.45 (s, 3 H, CH_3); MS *m/z* 310 (89), 295 (9), 191 (100), 162 (10); HRMS (EI 70 eV) calcd for $C_{19}H_{18}O_4$ *m/z* 310.1205 (M^+), found 310.1199.

3',4',7,8-Tetramethoxyflavone (4b): IR 1630 cm^{-1} (C=O); ^1H NMR δ 7.95 (d, J = 9 Hz, 1 H, 5-ArH), 7.62 (dd, J_a = 9 Hz, J_b = 2 Hz, 1 H, 6'-ArH), 7.44 (d, J = 2 Hz, 1 H, 2'-ArH), 7.04 (d, J = 9 Hz, 1 H, 6-ArH), 7.00 (d, J = 9 Hz, 1 H, 5'-ArH), 6.70 (s, 1 H, COCH), 4.04 (s, 3 H, OCH_3), 4.00 (s, 3 H, OCH_3), 3.98 (s, 3 H, OCH_3), 3.96 (s, 3 H, OCH_3); MS *m/z* 342 (100), 327 (6), 191 (4), 152 (7), 121 (4); HRMS (EI 70 eV) calcd for $C_{19}H_{18}O_6$ *m/z* 342.1103 (M^+), found 342.1111.

Pharmacology. The compounds were dissolved in dimethyl sulfoxide (1%) for *in vitro* experiments or acetone for the ear edema test. Synovial human recombinant phospholipase A_2 was a gift from D. J. Masters, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. [9,10-^{3}H]Oleic acid and radioimmunoassay kits for eicosanoids were purchased from Du Pont (Itisa, Madrid, Spain). All other reagents were from Sigma Chemical Co. (St. Louis, MO). Statistical analysis was performed by using Dunnett's test for multiple comparisons.

Lipid Peroxidation. Peroxidation was induced in rat liver microsomal fractions by three different systems: $FeSO_4$ (5 μM) + ascorbate (500 μM); 0.2 mM $NADP^+$, 4 mM glucose-6-phosphate, 0.6 unit glucose-6-phosphate dehydrogenase + CCl_4 (0.02 M), or 1.7 mM ADP, 100 μM Fe^{3+} + NADPH (400 μM) as previously described.[24] Incubations were performed in the presence of different concentrations of test compounds, and appropriate controls were used to discard any possible interference with the thiobarbituric acid assay.[25]

Free Radical Scavenging. Superoxide, hydroxyl, and peroxyl radicals were chemically generated following methods previously described (nitroblue tetrazolium reduction, deoxyribose degradation, and lysozyme inactivation assays, respectively).[24,26]

Phospholipase A_2 Assay. Phospholipase A_2 was assayed by using [^{3}H]oleate-labeled membranes of *Escherichia coli*, following a modification of the method of Franson et al.[27,28] Three secretory enzymes were assayed, *Naja naja* venom enzyme, hr-PLA_2, and bee venom enzyme.

Superoxide Generation by Human Neutrophils. Human neutrophils were purified by ficoll-hypaque sedimentation. The cells were resuspended in PBS containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}. More than 95% of the leukocytes were polymorphonuclear neutrophils with viability greater than 95% (trypan blue exclusion test). Aliquots of 0.5 mL neutrophils (2.5×10^6 cells/mL) were preincubated for 5 min at 37 °C with test compounds or the vehicle. TPA (1 μM) was the stimulus for superoxide generation, which was detected by reduction of cytochrome *c* at 550 nm.[28]

Elastase Release by Human Neutrophils. A total of 2.5×10^6 neutrophils/mL were preincubated with test compound or vehicle as above and then incubated with TPA (1 μM) for 10 min at 37 °C. After centrifugation at 1200*g* at 4 °C, supernatants were incubated with *N*-(*tert*-butoxycarbonyl)-L-alanine *p*-nitrophenyl ester (200 μM) for 10 min at 37 °C, and absorbances were determined at 414 nm in a microtiter plate reader.

Cytotoxicity Studies in Human Neutrophils. The cytoplasmic marker enzyme lactate dehydrogenase was determined by measuring the rate of oxidation of NADH.[29]

Eicosanoid Generation. A mixed suspension of human neutrophils (5×10^6/mL) and platelets (5×10^6/mL) was preincubated with test compounds or vehicle and then stimulated with A23187 (1 μM) for 10 min at 37 °C. After centrifugation as above, radioimmunoassays for LTB_4 and TXB_2 were performed in supernatant aliquots.

Mouse Ear Edema. TPA (2.5 μg) dissolved in 20 μL of acetone was applied to both inner and outer surfaces of the right ear of Swiss mice (20–25 g). Test compounds were applied topically in acetone before TPA. The left ear received only acetone. After 4 h the animals were killed by cervical dislocation, and equal sections of both ears were punched out and weighed to measure edema.[30]

Statistical Studies. Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's test.

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