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# EFFECTS OF FLAVONOIDS ON THE RELEASE OF REACTIVE OXYGEN SPECIES BY STIMULATED HUMAN NEUTROPHILS

## MULTIVARIATE ANALYSIS OF STRUCTURE–ACTIVITY RELATIONSHIPS (SAR)

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**Abstract**—In the present study we measured the inhibition by 34 compounds, either flavonoids or related substances, of the release of reactive oxygen species by human neutrophils after stimulation by three agents: the bacterial peptide *N*-fMetLeuPhe (FMLP), the protein kinase C activator phorbol myristate acetate (PMA) or opsonized zymosan (OZ), using two chemiluminescent probes, lucigenin or luminol in the presence or absence of horseradish peroxidase (HRP). The data matrix (34 × 7) was submitted to multivariate analysis: first, a correspondence factorial analysis to uncover levels of correlation among the biochemical parameters and the specificity of action of the test-compounds and second, a minimum spanning tree analysis that classified the chemical structures into a network describing both specificity and amplitude of the inhibition of the chemiluminescence response. The major conclusions of the analyses were: (a) opposition between inhibition of poly-morphonuclear leukocytes (PMNs) stimulated by FMLP and of PMNs stimulated by PMA or OZ implying that, for the molecules under study, there was a fundamental difference in the manner in which this inhibition occurred and, conversely, a difference in the nature of the stimulatory action of these activators. Molecules lacking hydroxyl groups on ring B, i.e. chrysin, chalcone, flavone and galangin, molecules glycosylated in position 7, i.e. hesperidin and naringin and ring B mono-hydroxylated molecules were, for the most part, at the origin of this dichotomy and might interfere with the membrane FMLP receptor; (b) a marked difference in chemiluminescence inhibition in the presence or absence of HRP that can be explained by the differential action of catechins compared to flavone and flavonol derivatives; (c) a similarity in biological profile between non-flavonoids such as chalcone and phloretin and low mean-activity flavonoids such as chrysin and galangin and between the non-flavonoid curcumin and the highly active flavonoid isorhamnetin; (d) a reaffirmation of the importance of ring A (C 5,7) and ring B (C 3′,4′) dihydroxylation, ring C (C 3) hydroxylation, but also of the presence of a methoxy group on ring B in engendering high potency. This potency is generally decreased by $C_2$–$C_3$ saturation and by glycosylation. The most active molecules identified in this study provide valuable information for the selection of simpler molecules (e.g. metabolites accounting for the potency of orally administered flavonoids) for further structure–activity relationship (SAR) studies that could lead to the design of novel drugs or prodrugs.

Flavonoids are a group of naturally-occurring phenolic compounds of the plant kingdom that are widespread in the human diet (daily levels of close to 1 g) and that are found in many plants traditionally used for their vascular protective properties. A wide variety of other activities have also been attributed to flavonoids such as, for example, anti-inflammatory, antimicrobial, anthelminthic, anti-allergic, hepato-protective, antithrombotic, antihormonal (versus growth hormone secretion) and antineoplastic activities (for reviews, see Refs 1–3). Some flavonoids possess cytotoxic, mutagenic and/or carcinogenic activities.

Particular importance has been accorded to the antioxidant or oxygen free-radical scavenging action and the enzyme-inhibitory actions of flavonoids which could account for many of the above pharmacological activities. In the organism, oxygen radicals come from metabolic sources but also from external environmental agents. The major sources are superoxide anion ($O_2^{\cdot-}$ §) and hydrogen peroxide ($H_2O_2$) generated during catabolism and during the respiratory burst from phagocytosis after viral or bacterial infection or the inflammation reaction. According to the cellular compartment in which the reactive oxygen species (ROS) are generated, either

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§ Abbreviations: BSA, bovine serum albumin; CFA, Correspondence Factorial Analysis; DMSO, dimethylsulfoxide; FMLP, *N*-fMetLeuPhe (*N*-formylmethionyl-leucylphenylalanine); HRP, horseradish peroxidase; lum, luminol; luc, lucigenin; MST, minimum spanning tree; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; OZ, opsonized zymosan; PKC, protein kinase C; PMA, phorbol myristate acetate; PMNs, poly-morphonuclear leukocytes; ROS, reactive oxygen species; SAR, structure–activity relationships; SOD, superoxide dismutase; DAG, diacylglycerol; AC, absolute contribution; RC, relative contribution; MPO, myeloperoxidase.

lipid peroxidation (in cell membranes) or damage to DNA and other macro-molecules (in a hydrophilic environment) predominates. This damage has been implicated in aging and in degenerative diseases such as cancer and atherosclerosis [4, 5]. The generation of ROS, particularly of $O_2^-$ and hydroxyl radical ($OH^\cdot$), is also a critical event in tumor promotion [6].

Many structure–activity studies have been performed on series of flavonoids using either oxygen radical scavenging activity or enzyme inhibition as end-points [7–15]. Some have also investigated the ability of flavonoids to generate free radicals [7, 8, 16]. The antioxidant properties of flavonoids can be assessed *in vitro* by measuring their ability to inhibit the production of ROS by stimulated polymorphonuclear neutrophil leukocytes (PMNs) [17, 21]. In the present extensive study, we have systematically screened a series of 28 flavonoids and of six structurally-related compounds on the production of ROS under three conditions of PMN stimulation, i.e., by the tumor-promoting agent phorbol myristate acetate (PMA) [also known as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)], by the potent chemoattractant peptide *N*-fMetLeuPhe (FMLP), and by opsonized zymosan (OZ), a particulate material phagocyted by PMNs. Although other stimulants are available (arachidonic acid, leukotrienes, ionophores, etc), we chose PMA because of the highly reproducible response it procures and FMLP and OZ because they are relevant to the physiopathological conditions encountered in phagocytosis.

Depending on the stimulating agent, PMN activation would appear to occur via different signalling pathways and generate a heterogeneity of ROS. In PMA-stimulated human PMNs, oxygen radical production correlates with protein kinase C (PKC) activation [22]. PKC from rat brain, activated by PMA or by the endogenous activator, diacylglycerol (DAG), is inhibited by plant flavonoids in a concentration-dependent manner according to flavonoid structure [10]. The PMN stimulating agent FMLP interacts with a specific plasma membrane receptor belonging to the G-protein-coupled receptor superfamily [23]. This interaction activates phospholipases C and D, stimulates the breakdown of phosphatidyl inositol 4,5-bisphosphate ($PIP_2$), and results in the transient accumulation of a small subset of highly polar inositol lipids and of DAG [24, 25]. Phosphoinositide phosphorylation is inhibited by quercetin [26]. Stimulation of PMNs by OZ differs from FMLP stimulation in that the breakdown of $PIP_2$ and the magnitude of DAG generation is greater with OZ than FMLP and the evaluation in $IP_3$ and DAG is sustained and not transient [27].

ROS generation was measured by two chemiluminescent probes, lucigenin (luc) and luminol (lum). Relaxation of the electronically-activated radicals to a ground state generates chemiluminescence caused by release of a photon. However, the origin of the emission within the heterogeneous pool of ROS cannot be identified with ease. It is generally accepted that luc measures the production of the first radical, $O_2^-$, which originates from the one-electron reduction of molecular oxygen by a membrane-bound NADPH-oxidase which is activated, at least in part, by PKC [28]. Lum in the presence of horseradish peroxidase (HRP) [29] can be used to specifically measure the formation of the next ROS, i.e. $H_2O_2$, generated to superoxide dismutase (SOD), since the HRP added in excess overrides the myeloperoxidase (MPO) present and, consequently, suppresses all the species subsequent to $H_2O_2$ formation. Finally, lum alone, in the absence of HRP, measures all ROS with varying sensitivity and in particular those generated by the MPO–$H_2O_2$–$Cl^-$ system.

In order to analyse the data obtained on the 34 compounds tested at two concentrations under seven experimental conditions, we have used multivariate statistical methods such as correspondence factorial analysis (CFA) [30–32] and minimum spanning tree analysis (MST) [33, 34] that highlight the relationships not only among the test-compounds, but also between the compounds and test conditions, and among the chosen conditions themselves. We have already applied these methods to the study of structure–activity relationships (SAR) of steroids [35–37], triphenylethylene derivatives [38–41] and antiparasitic agents [42, 43]. In the present study, we have used these methods to analyse the screening data on flavonoids in order to deduce SAR information on the putative mechanisms of action of the compounds, and further information on the significance of the tests.

## MATERIALS AND METHODS

### *Chemicals and test-compounds*

Lum, luc, HRP, bovine serum albumin (BSA), zymosan, PMA, and FMLP were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The RPMI medium was from Gibco (Grand Island, NY, U.S.A.) and Percoll from Pharmacia (Uppsala, Sweden). Flavone and catechin were obtained from Fluka (Buchs, Switzerland); flavane, flavanone, hesperidin methyl chalcone, and epicatechin from Koch Light (Colnbrook, U.K.), and all other flavonoids from Extrasynthèse (Genay, France). All flavonoids were of the highest commercially available purity. Other chemicals (RP grade) were from Merck (Darmstadt, F.R.G.).

The structural formulae of the 28 test-flavonoids and of six related compounds are given in Fig. 1. Each compound was solubilized in $10^{-2}$ M dimethylsulfoxide (DMSO) and used at final concentrations of $10^{-5}$ and $10^{-6}$ M.

### *Preparation of stimulating agents and of chemiluminescent probes*

PMA and FMLP ($10^{-2}$ M) were solubilized in DMSO and ethanol, respectively to give stock solutions used at a final $10^{-7}$ M concentration which was, under our assay conditions, the lowest concentration combining high sensitivity (a good chemiluminescence signal) and reproducible inhibition results, especially in the case of luc which often gave a weak signal that was, moreover, dependent upon the donor. Zymosan was opsonized (OZ) with fresh human serum and used at a 100 $\mu$g/mL concentration. The final concentration of lum

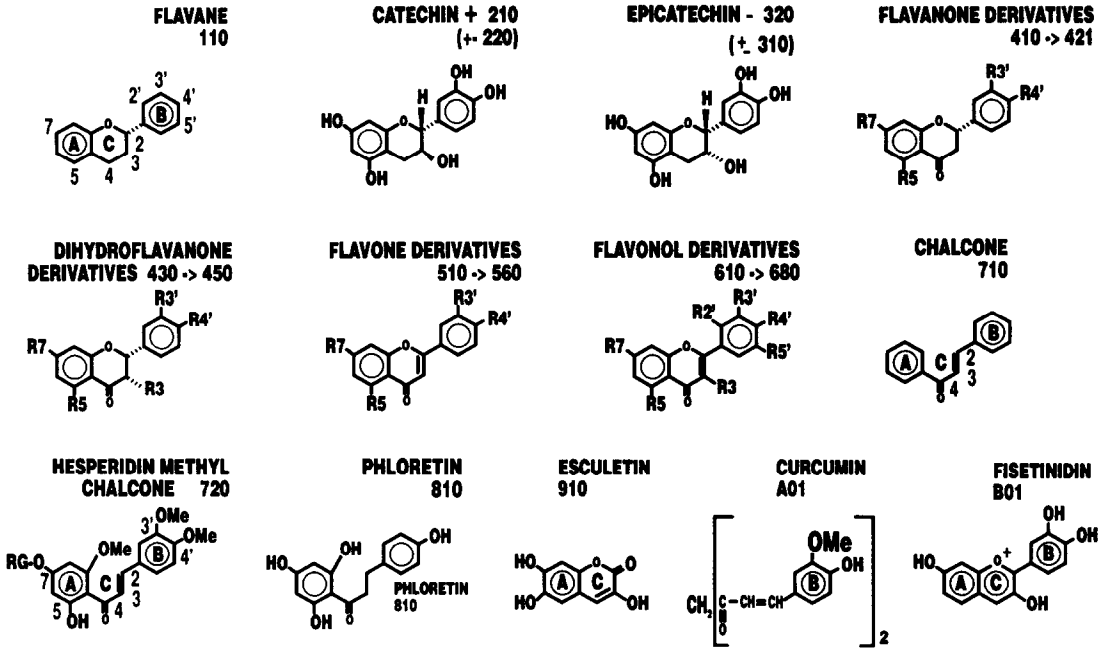



Fig. 1. General structural formulae of the test-flavonoids and of related compounds. (The rings of the related compounds are numbered unconventionally to facilitate comparison with flavonoids.)

in a 1% aqueous BSA solution and of luc in aqueous solution was $10^{-4}$ M.

*Isolation of PMNs* [44, 45]

Blood samples were taken from healthy adult donors after venipuncture and collected in heparinized tubes. PMNs were separated by centrifugation on a Percoll gradient as follows. A 5 mL solution of 63% Percoll in 0.15 M NaCl was layered onto a 5 mL solution of 72% Percoll and 5 mL of total blood were deposited on top. The samples were centrifuged at 400 *g* for 20 min at 4°, after which the PMNs were clustered at the 72/63% interface. The cell-layer was then removed and the cells were washed in Dulbecco's modified Eagle's medium (DMEM) medium. The erythrocyte fraction of the cell pellet was lysed by incubating for 10 min in a 130 mM $NH_4Cl$, 10 mM Tris, 16 mM $K_2CO_3$ buffer at pH 7.4. PMNs were collected after centrifugation. The purity of the cell suspension was at least 97% as assessed by May–Grunwald–Giemsa staining and the viability of the cells was at least 98% as assessed by Trypan blue exclusion.

*Chemiluminescence assay* [46, 47]

For each determination, we used $3 \times 10^5$ cells suspended in 500 $\mu$L of phosphate-buffered saline (PBS) containing either luc or lum, with or without 1 mU/mL HRP, in the present or absence of $10^{-5}$ or $10^{-6}$ M test-compound. Samples were maintained for 5 min at 37° under stirring in an LKB Wallac luminometer (model 1251) connected to an Apple II microcomputer. Chemiluminescence, expressed in mV/3 $\times$ $10^5$ cells, was measured before stimulation to obtain a basal value and then, after a 10 sec integration interval and a 10 sec waiting-period, at five time-intervals over a period of 100 sec after addition of the stimulating agent ($10^{-7}$ M) PMA or FMLP, 100 mg/mL OZ). The inhibitory effects of each test-compound were determined at the chemiluminescence peak (maximum value minus basal value) and expressed as percent control. Activation of response was seldom observed and was expressed as a negative inhibition value.

*Statistical analysis*

The data obtained in the present study represent a $34 \times (7 \times 2)$ matrix (effects of 34 flavonoids in seven tests at two concentrations). We submitted the data obtained at the $10^{-5}$ M concentration to multivariate analysis, as previously described for other matrices [35–43], in order to derive a maximum amount of information regarding the relationships among the variables, i.e. among the compounds themselves, among the test conditions and between the compounds and test conditions.

The statistical analyses were performed without data transformation except in the case of Fig. 4 where each data column was split into two subcolumns corresponding, on the one hand, to the experimental inhibition values and, on the other, to 'antivalues' (not shown) obtained by subtracting these experimental values from the maximum possible responses. For example, for test-compound No. 210 in Table 1, the luc antivalue is 100 and the PMAlum antivalue is 85. Splitting values enables to take into account not only the specificity of response of a molecule with respect to the tests but also the amplitude of the measured effects. Furthermore, although a few molecules activated rather than inhibited response, we allocated a 0.1% value to these molecules, and likewise to the inactive

Table 1. Percentage inhibition by two test-compound concentrations of the *in vitro* response of neutrophils to stimulation by PMA, FMLP, or OZ

| No. | Compound | 2'OH | 3'OH | 4'OH | 5'OH | 3OH | 5OH | 7OH | Concn (M) | PMAlum | PMAlum + H | FMLPlum | FMLPlum + H | Ozlum | Ozlum + H | PMAluc |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **110** | Flavane | H | H | H | H | H | H | H | $10^{-5}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **210** | Catechin + | H | OH | OH | H | OH | OH | OH | $10^{-5}$ | 15 | 69 | 24 | 62 | 27 | 69 | 0 |
| | | | | | | | | | $10^{-6}$ | 7 | 50 | 0 | 63 | 0 | 16 | 0 |
| **220** | Catechin +− | H | OH | OH | H | OH | OH | OH | $10^{-5}$ | 19 | 92 | 19 | 80 | 28 | 64 | 0 |
| | | | | | | | | | $10^{-6}$ | 8 | 36 | 0 | 75 | 10 | 26 | 0 |
| **310** | Epicatechin +− | H | OH | OH | H | OH | OH | OH | $10^{-5}$ | 27 | 92 | 27 | 85 | 34 | 68 | 0 |
| | | | | | | | | | $10^{-6}$ | 25 | 42 | 7 | 71 | 0 | 12 | 0 |
| **320** | Epicatechin − | H | OH | OH | H | OH | OH | OH | $10^{-5}$ | 30 | 93 | 20 | 88 | 35 | 75 | 0 |
| | | | | | | | | | $10^{-6}$ | 19 | 43 | 8 | 85 | 9 | 0 | 0 |
| **410** | Flavanone | H | H | H | H | H | H | H | $10^{-5}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **420** | Naringenin | H | H | OH | H | H | OH | OH | $10^{-5}$ | 9 | 0 | 38 | 17 | 17 | 17 | 10 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 23 | 19 | 0 | 0 | 0 |
| **421** | Naringin | H | H | OH | H | H | OH | O-GR | $10^{-5}$ | 0 | 0 | 0 | 20 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 32 | 0 | 0 | 0 |
| **430** | Hesperetin | H | OH | O-Me | H | H | OH | OH | $10^{-5}$ | 53 | 85 | 45 | 56 | 53 | 57 | 0 |
| | | | | | | | | | $10^{-6}$ | 15 | 40 | 18 | 25 | 22 | 26 | 0 |
| **431** | Hesperidin | H | OH | O-Me | H | H | OH | O-GR | $10^{-5}$ | 0 | 0 | 0 | 13 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **440** | Fustin | H | OH | OH | H | OH | H | OH | $10^{-5}$ | 23 | 91 | 24 | 90 | 13 | 36 | 0 |
| | | | | | | | | | $10^{-6}$ | 12 | 37 | 0 | 83 | 0 | 19 | 0 |
| **450** | Taxifolin | H | OH | OH | H | OH | OH | OH | $10^{-5}$ | 36 | 89 | 34 | 88 | 50 | 70 | 0 |
| | | | | | | | | | $10^{-6}$ | 22 | 43 | 21 | 68 | 26 | 27 | 0 |
| **510** | Flavone | H | H | H | H | H | H | H | $10^{-5}$ | 0 | 0 | 20 | 19 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 8 | 5 | 0 | 0 | 0 |
| **520** | Chrysin | H | H | H | H | H | OH | OH | $10^{-5}$ | 0 | 5 | 28 | 19 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| **530** | Apigenin | H | H | OH | H | H | OH | OH | $10^{-5}$ | 19 | 0 | 10 | 46 | 0 | 14 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 10 | 35 | 0 | 0 | 0 |
| **540** | Acacetin | H | H | O-Me | H | H | OH | OH | $10^{-5}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **550** | Luteolin | H | OH | OH | H | H | OH | OH | $10^{-5}$ | 73 | 94 | 90 | 89 | 63 | 78 | 28 |
| | | | | | | | | | $10^{-6}$ | 17 | 14 | 18 | 52 | 13 | 0 | 0 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **560** | Diosmetin | H | OH | O-Me | H | H | OH | OH | $10^{-5}$ | 81 | 69 | 82 | 69 | 74 | 67 | 22 |
| | | | | | | | | | $10^{-6}$ | 59 | 39 | 50 | 32 | 36 | 16 | 0 |
| **610** | Galangin | H | H | H | H | OH | OH | OH | $10^{-5}$ | 0 | −37 | 78 | 78 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | −27 | −46 | 23 | 0 | 0 | 0 | 0 |
| **620** | Kaempferol | H | H | OH | H | OH | OH | OH | $10^{-5}$ | 67 | 0 | 45 | 87 | 51 | 64 | 14 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 0 | 71 | 5 | 0 | 0 |
| **630** | Fisetin | H | OH | OH | H | OH | H | OH | $10^{-5}$ | 35 | 55 | 47 | 81 | 44 | 64 | 0 |
| | | | | | | | | | $10^{-6}$ | −8 | 33 | 12 | 59 | 11 | 31 | 0 |
| **640** | Quercetin | H | OH | OH | H | OH | OH | OH | $10^{-5}$ | 51 | 90 | 34 | 55 | 45 | 61 | 10 |
| | | | | | | | | | $10^{-6}$ | 28 | 37 | 18 | 23 | 20 | 19 | 0 |
| **641** | Quercitrin | H | OH | OH | H | O-R | OH | OH | $10^{-5}$ | 0 | 34 | 39 | 86 | 34 | 69 | 13 |
| | | | | | | | | | $10^{-6}$ | 10 | 29 | 11 | 89 | 0 | 46 | 0 |
| **642** | Rutin | H | OH | OH | H | O-GR | OH | OH | $10^{-5}$ | 54 | 90 | 46 | 85 | 51 | 81 | 0 |
| | | | | | | | | | $10^{-6}$ | 30 | 64 | 59 | 73 | 27 | 53 | 0 |
| **650** | Rhamnetin | H | OH | OH | H | OH | OH | O-Me | $10^{-5}$ | 89 | 94 | 73 | 67 | 83 | 75 | 35 |
| | | | | | | | | | $10^{-6}$ | 24 | 26 | 65 | 47 | 12 | 14 | 10 |
| **660** | Isorhamnetin | H | O-Me | OH | H | OH | OH | OH | $10^{-5}$ | 96 | 96 | 95 | 92 | 91 | 54 | −190 |
| | | | | | | | | | $10^{-6}$ | 13 | 5 | 74 | 58 | 18 | 8 | −89 |
| **670** | Morin | OH | H | OH | H | OH | OH | OH | $10^{-5}$ | 45 | 0 | 26 | 58 | 34 | 0 | 10 |
| | | | | | | | | | $10^{-6}$ | 18 | 0 | 14 | 22 | 0 | 0 | 0 |
| **680** | Myricetin | H | OH | OH | OH | OH | OH | OH | $10^{-5}$ | 45 | 50 | 18 | 5 | 42 | 38 | 31 |
| | | | | | | | | | $10^{-6}$ | 25 | 8 | 0 | 0 | 9 | 4 | 0 |
| **710** | Chalcone | H | H | H | H | H | H | H | $10^{-5}$ | 0 | 0 | 71 | 60 | 4 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 12 | 5 | 0 | 0 | 0 |
| **720** | Hesperidin methyl chalc | H | O-Me | O-Me | H | H | OH | O-GR | $10^{-5}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 0 | 15 | 21 | 15 | 0 | 0 |
| **810** | Phloretin | H | H | OH | H | H | OH | OH | $10^{-5}$ | 14 | 0 | 32 | 0 | 10 | 0 | 0 |
| | | | | | | | | | $10^{-6}$ | 5 | 0 | 7 | 0 | 5 | 0 | 0 |
| **910** | Esculetin | | | | | | | | $10^{-5}$ | 43 | 66 | 55 | 56 | 51 | 66 | 20 |
| | | | | | | | | | $10^{-6}$ | 0 | 31 | 0 | 0 | 0 | 0 | 0 |
| **AO1** | Curcumin | | | | | | | | $10^{-5}$ | 86 | 79 | 91 | 85 | 85 | 69 | −67 |
| | | | | | | | | | $10^{-6}$ | 24 | 29 | 66 | 46 | 32 | 10 | 0 |
| **BO1** | Fisetinidin | H | OH | OH | H | OH | H | OH | $10^{-5}$ | 19 | 65 | 25 | 49 | 17 | 58 | 0 |
| | | | | | | | | | $10^{-6}$ | 0 | 37 | 0 | 0 | 0 | 20 | 0 |

Stimulation was measured using either luc or lum as chemiluminescence probes in the presence (+H) or absence of HRP.

The values are the means of duplicates obtained in at least two experiments. The left-hand panels indicate the positions of hydroxy and methoxy (OMe) groups on rings B, C and A of the flavonoids (OR: *o*-rhamnose, OGR: *o*-rutinose).

molecules, since this introduces a lesser bias than the use of zero values.

To compare the profiles of the variables, we used the Chi²-metric to convert the data matrix to a distance matrix which was analysed by both a factorial approach and by an automatic classification procedure. The chosen methods were CFA [30–32, 35, 41] and the MST method [33, 39, 40, 42, 43].

*CFA.* The measurements obtained for a population of molecules assessed on a set of tests engender a cloud of points depicting both molecules and tests in an *N*-dimensional space. To represent these visually within a 2D-space that best accounts for the total information (variance) with minimum deformation, it is necessary to define a rank order of orthogonal or *factorial* axes.

CFA consists in treating the data matrix as follows: (a) applying the Chi²-metric to obtain a distance matrix, (b) calculating the Eigenvalues and Eigenvectors of this matrix to obtain the rank order of the factorial axes, (c) calculating the relative (RC) and absolute (AC) contributions of each initial variable to the main factorial axes in order to establish their significance, (d) selecting factorial axes to draw 2D-maps.

The per cent variance (information) associated with a given factorial axis is denoted $\tau$. A factorial axis is generated by the ACs of the variables to this axis ($\Sigma AC = 100\%$). Each variable is, however, distributed over the number of factorial axes needed to describe the total variance of the system and has a RC to each axis, the sum total of which for all factorial axes equals 1 ($\Sigma RC = 1$). The first factorial map ($\varphi_1\varphi_2$) is a description of those molecules and tests that are most discriminatory within the study and embodies most of the information. The second map ($\varphi_3\varphi_4$) is defined by variables of lesser variance and, even though it embodies less information, this information has nevertheless the same significance value as that derived from the first factorial map.

The main advantages of CFA are that single factorial maps describe correlations among all measured variables, i.e. both molecules and tests can be represented on the same maps, and that these maps uncover the strongest correlations from amidst information of lesser import. CFA maps are therefore particularly well suited to the analysis of SAR. Furthermore, CFA can be used to create model systems to which an *N*th + 1 test or compound can be added. Thus in Fig. 2 the data for $10^{-6}$ M of substance, although poorer in information than the $10^{-5}$ M data is often equal to zero, were introduced into the initial analysis as additional variables (see arrows) and confirmed the strength of the original correlations.

*MST method.* The Prim algorithm [48] was applied to a Chi²-distance matrix made up of the transformed ("split") values (see above) to create an MST describing both specificity and amplitude of response [39].

*Calculations.* Calculations were performed on a 80286 + 80287 microcomputer with a program adapted for BASIC from FORTRAN Anacor software. The MST program and a simplified version of the CFA program for running on an IBM PC compatible computer are available upon request from J.C. Doré (Muséum National d'Histoire Naturelle, 63 rue Buffon, 75005 Paris, France). The present CFA analysis was performed using a statistical package obtained from ITCF (8 ave du Président Wilson, 75116 Paris, France).

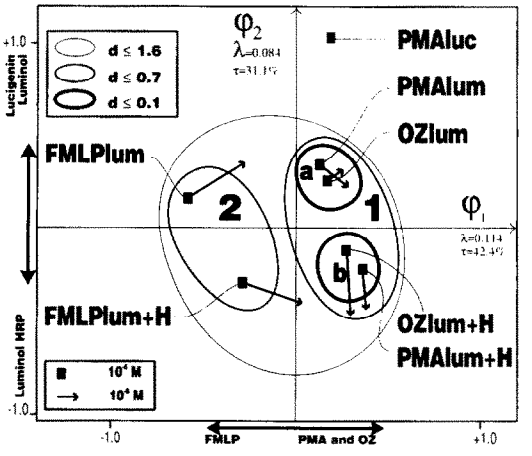



Fig. 2. $\varphi_1\varphi_2$ factorial map of the $10^{-5}$ M data in Table 1. Only the locations of the variables describing experimental conditions (■), and not of the test-compounds, are shown. Affiliations are denoted by the relative positions of the variables and by contours (1a, 1b, 2) drawn on the basis of a hierarchical classification analysis (not shown). The darker the shading, the closer the relationship. The positions of the variables after introduction of the $10^{-6}$ M data in Table 1 as supplementary variables into the factorial analysis are given by the tips of the arrows. [$\lambda$: Eigenvalue obtained by diagonalization of the symmetric matrix; $\tau$: per cent variance (information) associated with a given factorial axis.]

## RESULTS

### *Inhibition of neutrophil stimulation. Examination of data prior to multivariate analysis*

The percentage *in vitro* inhibition by the 28 flavonoids and by six structurally-related substances of the response of neutrophils when stimulated by various agents (PMA, FMLP, OZ) is given in Table 1. Broadly, the test-compounds can be classified as follows (see also Fig. 1). Five derivatives of flavane and flavan-3-ol (flavanol) (**110–320**), seven of flavanone and flavanon-3-ol (dihydroflavonol) (**410–450**), six of flavone (**510–560**), and 10 of flavonol (**610–680**). The presence of hydroxy group substituents is indicated in the left-hand panel of the table. The structurally-related compounds are three chalcone derivatives (**710–810**), one coumarin derivative (**910**), one bis-phenyl (**A01**) and one anthocyanidin (**B01**) whose formulae have been numbered unconventionally in Fig. 1 in order to facilitate comparison with the flavonoids.

Either lum or luc was used as a chemiluminescent probe. When using lum, PMNs were stimulated by one of three agents, either PMA, FMLP or OZ, in the presence (+H) or absence of HRP which specifically enhanced $H_2O_2$ luminescence. When

using luc, which is less sensitive than lum, the cells were stimulated by PMA only since PMA gives the most abundant and reproducible response. Thus, for each test-compound, seven assays were performed denoted as follows: PMAlum, PMAlum + H, FMLPlum, FMLPlum + H, OZlum, OZlum + H and PMAluc.

Although such voluminous data are difficult to interpret *de visu*, it appears that a hydroxy group on the skeleton is necessary for inhibition. Two exceptions are the 4-keto compounds with a $C_2$–$C_3$ double bond, chalcone (**710**) and flavone (**510**), which inhibited FMLP-stimulation of cells.

*Enhancement versus inhibition of chemiluminescence.* A most surprising observation was that, under certain conditions, three test-compounds enhanced rather than inhibited response. Galangin (**610**), the only flavonol with an unsubstituted B ring (compare with the flavones **510**, **520**) enhanced PMA stimulation of the lum response in the presence or absence of HRP (up to 45%). Isorhamnetin (**660**) and curcumin (**A01**), which are the only molecules with 4′-hydroxy and 3′-methoxy groups on ring B, enhanced the PMA-induced luc response (by 190 and 67%, respectively at a $10^{-5}$ M concentration, by 89% for isorhamnetin at a $10^{-6}$ M concentration). Enhancement was consistent and reproducible in each case. On the other hand, the 8% enhancement by a $10^{-6}$ M concentration of fisetin can be considered negligible.

*Influence of probe.* In PMA-stimulated cells luc and lum responses differed widely. Whereas inhibition of the lum response was frequent, inhibition of the luc response by a $10^{-5}$ M concentration of test-compound was either unmeasurable (22/34 inhibitors tested) or very low (35% or less) with the notable exceptions of isorhamnetin (**660**) and curcumin (**A01**) (see above). At a $10^{-6}$ M concentration of test-compound, only rhamnetin and isorhamnetin were active (10% inhibition and 89% enhancement, respectively). HRP enhanced inhibition of the lum response particularly at the $10^{-6}$ M concentration (see **210**, **320**, **430**, **630** and **642**) but sometimes at the higher $10^{-5}$ M concentration with the exception of compounds **420**, **560**, **670**, **680**, **810** and **A01**. The active flavonoids are 5,7-diphenols (ring A) and have at least one hydroxy group on ring B.

*Influence of stimulating agent.* Whereas inhibitions of PMA- and OZ-stimulated responses were highly similar when assayed with lum with or without HRP, more marked inhibitions of FMLP-stimulation were often observed, for instance by molecules **510**, **520**, **610** and **710** which have no substituent on ring B. These molecules are therefore not solely scavengers.

*Influence of glycosylation.* Flavonoids glycosylated in position 7 [naringin (**421**) and hesperidin (**431**)] were weak inhibitors of chemiluminescence under conditions of stimulation by FMLP and enhancement by lum + HRP in stark contrast with the behavior of the corresponding aglycones (**420** and **430**) and with that of flavonoids glycosylated in position 3 (**642** and **641**) which exhibited much more pronounced inhibitory activity under all assay conditions.

*Multivariate analysis of SAR*

Unbiased SAR cannot be drawn from a data table such as Table 1 that gives the results for 34 compounds (rows) at two concentrations for seven variables (columns) without resorting to a multivariate analysis. A CFA was therefore performed followed by a MST analysis. The CFA was carried out on untransformed data and highlighted the specificity of the inhibition exerted by the test-compounds toward the different stimulating agents and probes whereas the MST was derived from an analysis of the split data (see Materials and Methods) and classified the test-compounds according to specificity and amplitude of inhibition.

In our multivariate approach, we chose to analyse the data only at the $10^{-5}$ M concentration for two main reasons: first, in order to reduce the number of zero values which were more frequent at the $10^{-6}$ M concentration and second, in order to dispose of a higher molecule-to-variable ratio since the use of two concentrations implies the analysis of 14 as opposed to seven variables for 34 molecules. Decreasing the molecule-to-variable ratio weakens the power of the analysis. One can note from Table 1 that this decision was of major importance only in the case of hesperidin methyl chalcone (**720**), which is inactive at $10^{-5}$ M but a weak inhibitor of the FMLP-induced lum response at $10^{-6}$ M. The data obtained with the $10^{-6}$ M concentration were, however, not totally neglected since they were later introduced into the analysis as supplementary variables (see below). Furthermore, in view of the fact that three compounds [galangin (**610**), isorhamnetin (**660**) and curcumin (**A01**)] could enhance response at a $10^{-5}$ M concentration, it would have theoretically been possible to consider a stimulatory versus inhibitory response for each compound but this would have again unnecessarily multiplied the number of variables. We therefore chose to consider these compounds simply as inactive inhibitors.

*CFA of the variables describing experimental conditions.* Since the $\varphi_1\varphi_2$ factorial maps in Figs 2 and 3, which are superimposable, represent a very high proportion (73.5%) of the variance (information content) of the system, it is these figures that discriminate the tests and molecules best. For the sake of clarity, only the locations of the variables describing experimental conditions (stimulating agents and probes) are shown in Fig. 2; test-compounds have been voluntarily omitted. Conversely, Fig. 3 represents the test-molecules only.

The first step of a CFA is to attribute meaning to the factorial axes by examining the values and signs of the absolute and relative contributions of each variable to each axis (Table 2). In this instance, the $\varphi_1$ axis, which represents 42.4% of the information (see $\tau$ value), describes the influence of stimulating agents and distinguishes inhibition of PMNs stimulated by FLMP (left-hand quadrants) from those stimulated by either PMA or OZ (right-hand quadrants) regardless of whether luc or lum (with or without HRP) was used as a probe. The $\varphi_2$ axis ($\tau = 31.1\%$), on the other hand, relates to these

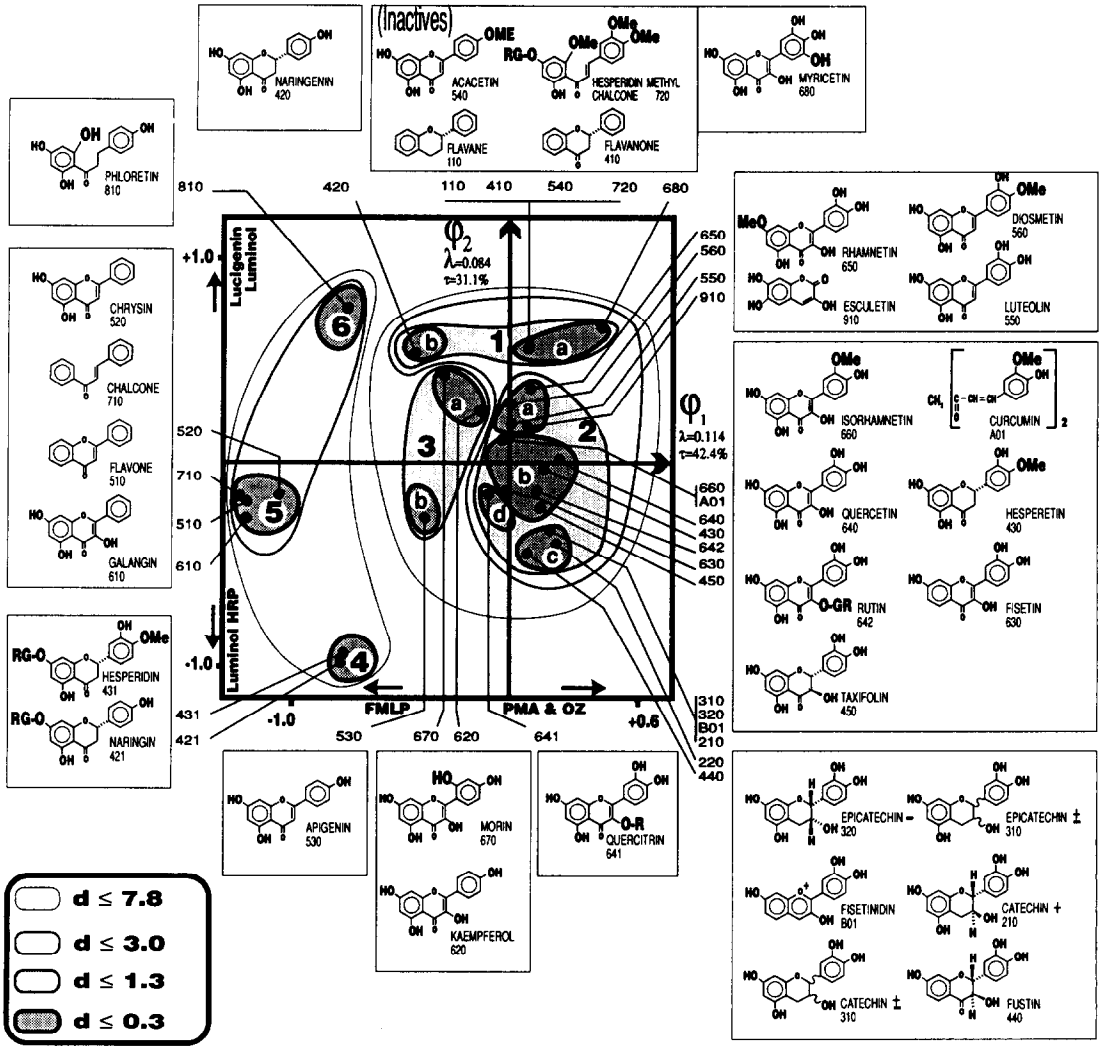



Fig. 3. $\varphi_1\varphi_2$ factorial map of the $10^{-5}$ M data in Table 1. Although only the locations of the test-compounds are shown, this map can be directly superimposed upon Fig. 2. As in Fig. 2, affiliation is represented by contours of different shade around clusters (numbered from 1 to 6).

Table 2. RC and AC contributions of the variables describing the experimental conditions to the factorial axes

| Variable | $\varphi_1$ Axis $\lambda = 0.014$ $\tau = 42.4\%$ | | | $\varphi_2$ Axis 0.084 31.1% | | | $\varphi_3$ Axis 0.030 11.3% | | | $\varphi_4$ Axis 0.026 9.6% | | | $\varphi_5$ Axis 0.012 4.4% | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S | RC | AC | S | RC | AC | S | RC | AC | S | RC | AC | S | RC | AC |
| PMAluc | + | 0.019 | 0.7 | + | 0.587 | 30.9 | − | 0.214 | 30.9 | + | 0.159 | 27.2 | + | 0.021 | 7.9 |
| PMAlum | + | 0.072 | 1.8 | + | 0.525 | 17.9 | + | 0.003 | 0.3 | − | 0.358 | 39.7 | + | 0.012 | 2.8 |
| PMAlum + H | + | 0.569 | 21.4 | − | 0.210 | 10.8 | + | 0.102 | 14.4 | + | 0.043 | 7.2 | + | 0.075 | 27.5 |
| FMLPlum | − | 0.798 | 46.2 | + | 0.059 | 4.7 | + | 0.103 | 22.5 | + | 0.038 | 9.8 | − | 0.001 | 0.7 |
| FMLPlum + H | − | 0.382 | 16.2 | − | 0.397 | 22.9 | − | 0.184 | 29.3 | − | 0.028 | 5.3 | + | 0.007 | 3.0 |
| OZlum | + | 0.220 | 3.2 | + | 0.510 | 10.0 | + | 0.013 | 0.7 | − | 0.082 | 5.3 | − | 0.042 | 5.8 |
| OZlum + H | + | 0.523 | 10.5 | − | 0.105 | 2.9 | − | 0.026 | 1.9 | + | 0.063 | 5.6 | − | 0.268 | 52.3 |

$\lambda$: Eigenvalue obtained by diagonalization of the symmetric matrix; $\tau$: per cent variance (information) associated with a given factorial axis.

probes and differentiates assay conditions, i.e. conditions when HRP was (lum + HRP) (bottom quadrants) or was not (luc, lum) (top quadrants) used.

In a factorial map, proximity implies affiliation, distance implies diversity. The $\varphi_1\varphi_2$ factorial map thus reveals that stimulation by FMLP (contour 2) stands in opposition to stimulation by PMA or OZ (contour 1) ($\varphi_1$ axis). In other words, the behavior of this population of molecules toward these stimulants of PMN activation differs in nature. The map also classifies the probes from top to bottom ($\varphi_2$ axis) in the order luc, lum without HRP, lum with HRP. It is interesting to note that this classification, in which lum without HRP lies in an intermediate position, corresponds to the specificity of these probes as described with regard to ROS detection: luc measures $O_2^{\cdot-}$ only; lum alone would measure a gamut of ROS (including $O_2^{\cdot-}$ + $OH^{\cdot}$ + OHCl + $ClO^-$ + $H_2O_2$) whereas lum plus HRP primarily measures $H_2O_2$. Since the two stimulating agents PMA and OZ are very close to each other, they must have great analogy as regards their reaction to this population of test-compounds. This analogy holds whether HRP is present (contour 1b) or not (contour 1a) in the assay medium.

It is possible in a CFA analysis to introduce additional data for any one variable at a time. The above CFA was therefore used as a model to introduce, sequentially for each variable describing the experimental conditions, the data corresponding to the $10^{-6}$ M concentration of test-compound (Table 1). The locations of the variables based on the $10^{-6}$ M data are indicated by the tips of the arrows in Fig. 2. On the whole, the relative positions of the variables are not greatly affected, except maybe for FMLP whether with (FMLPlum + H) or without (FMLPlum) HRP, possibly because these variables contribute little to this map. Thus, in spite of the zero or very low values obtained at the $10^{-6}$ M concentration that might introduce a statistical bias, these data nevertheless confirm the relationships derived from our above analysis of the $10^{-5}$ M inhibition data.

*Correspondence factorial analysis of the chemical variables. Specificity of response of the test-compounds*

From a discussion of the relationships among the variables selected to assay inhibition of PMN stimulation, we shall now turn to a study of the population of molecules that enabled us to define these relationships.

*(a) The $\varphi_1\varphi_2$ factorial map.* The positions of the molecules within the $\varphi_1\varphi_2$ factorial map are shown in Fig. 3. As this factorial map was derived by CFA, it is totally licit to superimpose it directly upon Fig. 2 and thus ascertain not only the lay-out of the molecules with respect to each other but also their position with respect to the variables describing experimental conditions. According to the absolute and relative contributions of these variables to the factorial axes given as in Table 2 and, as already explained above for Fig. 2, the left-hand quadrants are dominated by FMLP stimulation, the right-hand quadrants by PMA and OZ stimulation, the top quadrants by luc and lum, and the bottom quadrants by lum plus HRP. Contours based on a hierarchical clustering classification (not shown) have been drawn around molecules with similar behavior with regard to the experimental conditions in order to visualize closeness of affiliation. There are six main clusters of molecules numbered from 1 to 6, the larger clusters 1–3 being formed of subclusters denoted a–d. We shall first of all examine the specificity of non-flavonoids, then of the flavonoids themselves according to their C, B and A ring substituents.

*Specificity of non-flavonoids.* The first striking observation in Fig. 3 is that the behavior of non-flavonoids cannot be distinguished from that of flavonoids implying that it is not the flavone skeleton *per se* but rather the nature and spatial configuration of the substituents that determine activity. For instance, esculetin (**910**) is part of cluster 2a and curcumin (**A01**) is in the same position as isorhamnetin (**660**) at the periphery of cluster 2b. The sole common structural feature between these two molecules is the *meta*-methoxylated $C_2$–$C_3$ unsaturated *ortho*-diphenol. Furthermore, it is satisfying to note that, even though our analysis disregarded stimulation it nevertheless grouped together the two molecules, curcumin and isorhamnetin, which exert a stimulatory action on PMAluc.

As regards the specificity of action of chalcone (**710**) at a $10^{-5}$ M concentration, this molecule behaves very much like molecules of cluster 5 [the presence of galangin in this cluster is due to its strong inhibition of FMLP activation and to the fact that its ability to enhance response was not taken into account in the analysis (see above)]. On the other hand, hesperidin methyl chalcone (**720**), a methoxylated and glycosylated derivative, is in cluster 1a together with inactive compounds. Fisetinidin (**BO1**), a flavinium salt, is in cluster 2c together with catechins (**310**, **320**, **210** and **220**) and fustin (**440**). The only non-flavonoid compound that cannot be readily compared to flavonoids is phloretin (**810**) which is the sole member of cluster 6. Phloretin is also the only molecule that combines the following characteristics: lack of inhibition of the luc response, inhibition of the lum response in the absence of HRP only, and greater inhibition of responses stimulated by FMLP than by OZ or PMA.

*Specificity of response of flavonoids, ring C.* Three principal chemical modifications concern the C ring in our population of molecules; $C_2$–$C_3$ unsaturation, a keto group at $C_4$ and a hydroxyl group at $C_3$ which can be glycosylated as in quercitrin (**641**) (*o*-rhamnose) or rutin (**642**) (*o*-rutinose). These chemical modifications change the electronic conjugation of the skeleton and are used to classify flavonoids (flavane, flavanone, etc.).

According to Fig. 3, there is a progressive change in inhibition pattern towards increased specificity towards an FMLP-stimulated lum–luc response when moving from a structure with no C-ring unsaturation and no 4-keto group (bottom right-hand quadrant) to a structure with a combination of these two features (top left-hand quadrant). Catechin isomers (**210–320**) devoid of a 4-keto group are clustered in 2c with fustin (**440**), which has a 4-keto group, and,

unexpectedly, with fisetidinin (**B01**), which has an aromatic C ring. The keto-homolog taxifolin (**450**) of catechin is in cluster 2b. Specificity with regard to inhibition of FMLP-stimulation is modified by the introduction of a 3-hydroxy group. As already mentioned above, opening of the C ring, e.g. the non-flavonoid structures chalcone (**710**) and phloretin (**810**), generates molecules that behave like flavones and flavanones that are unsubstituted, or at most dihydroxylated on a ring other than B, as witnessed by their proximity to these molecules.

*Specificity of response of flavonoids, ring B.* Flavonoids with an unsubstituted B ring and an unsaturated C ring with a 4-keto substituent [i.e. flavone (**510**), chrysin (**520**), galangin (**610**), and chalcone (**710**)] are all grouped in cluster 5. They all specifically inhibit FMLP-stimulation whether HRP is or is not added to the probe and except for galangin, which unexpectedly also activates PMAlum with or without HRP, they possess no other activity.

As regards ring B hydroxylated derivatives, all 4′-hydroxy flavonoids devoid of a 3′-hydroxy group (**420**, **421**, **530**, **620**, **670** and **810**) are located in clusters or subclusters within the left-hand quadrants of the map signifying that, to a greater or lesser extent, they also have FMLP specificity (the position of **620**, for instance, which is close to the $\varphi_1$ axis, is characterized more by a lack of inhibition of PMAlum + H than inhibition of FMLP stimulation). 2′-Hydroxylation of kaempferol (**620**) to give morin (**670**) has little influence on profile (cluster 3a) whereas 3′-hydroxylation to give fisetin (**630**) (bottom right-hand quadrant) increases specificity toward PMA and OZ and also toward HRP enhancement. Myricetin (**680**) with 3′,4′,5′-hydroxy groups is an atypical molecule at the periphery of a cluster (1a) uniting inactive flavonoids.

*Specificity of response of flavonoids, ring A.* The chosen molecules yield information on hydroxylation in positions 7 or 5 of ring A but this information is sparse. The effect of the lack of a hydroxy group in 7 is difficult to gauge since only two active molecules, flavone (**510**) and chalcone (**710**), both characterized by an unsubstituted B ring, fall into this category (cluster 5 of Fig. 3). All the 7-hydroxylated flavonoids appear randomly situated around the origin of the $\varphi_1\varphi_2$ factorial map. The lack of a 5-hydroxy group on ring A when ring B is unsubstituted does not affect response pattern [see flavone (**510**) and chalcone (**710**) compared to chrysin (**520**) and galangin (**610**) in cluster 5].

*(b) The $\varphi_3\varphi_4$ factorial map and $\varphi_4$ axis.* According to Table 2, a substantial proportion (20.9%) of the total information content of the system is accounted for by the $\varphi_3$ and $\varphi_4$ axes. The $\varphi_3\varphi_4$ factorial map (not shown) describes the singular biological behavior of chemically atypical molecules but, in view of the limited number of these molecules, it is premature to draw any definite conclusions from these observations as from the $\varphi_5$ axis which incorporates 4.4% of the information and differentiates between inhibition of stimulation by PMA and OZ in the presence of HRP for a handful of molecules.

*MST analysis. Specificity and amplitude of response*

To account not only for specificity but also amplitude of response, it is necessary to consider split data as explained in the Materials and Methods. We applied the Prim algorithm [48] to the $Chi^2$-distance table derived from these data to construct a MST (Fig. 4). A MST describes how the system under study is structured by calculating the shortest route without the *N*-dimensional space, without loops or backtracking, that links the molecules into a network (analogous to Calder's mobile) on the basis of their responses in the tests. Adjacent molecules within the network have similar biological profiles with respect to the tests; distant molecules have dissimilar properties. In Fig. 4, the thickness of the branches reflects the degree of similarity in response under different experimental conditions; the height of the branches reflects the mean relative activity with respect to the most potent compound isorhamnetin (**660**). The individual activity profiles for the tip of the branch and intersection molecules are shown in a separate diagram.

*Potency of flavonoids, ring C.* A closed C ring is not a prerequisite for inhibition; chalcone (**710**) is close to chrysin (**520**) and galangin (**610**). The latter compound, however, has stimulatory activity which, as mentioned previously, was not taken into account in the analysis. Both chrysin and galangin have in common a 4-keto substituted and unsaturated C ring and an unsubstituted B ring. Phloretin (**810**) is situated between chrysin (**520**) and naringenin (**420**). Thus, opening of the C ring of naringenin results in only a small loss in activity.

A 4-keto substituent on ring C influences inhibitory potency little. The catechins (**210–320**) are situated just behind their keto-homolog taxifolin (**450**) along the same branch and display little loss in activity. The potency of the natural planar optical isomers, − epicatechin and + catechin, is comparable to that of the racemic compounds.

$C_2$–$C_3$ unsaturation of ring C increases the potencies of kaempferol (**620**) over naringenin (**420**) and of diosmetin (**560**) over hesperetin (**430**) but little influences the potencies of fisetin (**630**) compared to fustin (**440**) and of taxifolin (**450**) compared to quercetin (**640**). The introduction of a 3-hydroxy group decreases the potency of luteolin (**550**) [compare to quercetin (**640**)] but this observation cannot be generalized for lack of data on other compounds. 3-*o*-Glycosylation of quercetin (**640**) with *o*-rhamnose [quercitrin (**641**)] decreases potency, with *o*-rutinose [rutin (**640**)] increases potency.

*Potency of flavonoids, ring B.* Ring B hydroxylation markedly influences potency with increases in the order 3′,4′H→4′OH→3′,4′-OH as observed for galangin (**610**)→kaempferol (**620**)→quercetin (**640**) and also for chrysin (**520**)→apigenin (**530**)→luteolin (**540**). 4′-Methoxylation of 3′,4′-*ortho*-diphenol slightly decreases activity [compare diosmetin (**560**) and luteolin (**550**)] whereas a 4′-methoxy in the absence of a hydroxy group wipes out activity [compare acacetin (**540**) and apigenin (**530**)]. 3′-Methoxylation in the presence of a 4′-hydroxy group enhances activity [compare isorhamnetin (**660**) and quercetin (**640**) and see high potency of curcumin (**A01**)]. The introduction of a further hydroxy group, however, decreases activity as witnessed by the 2′-

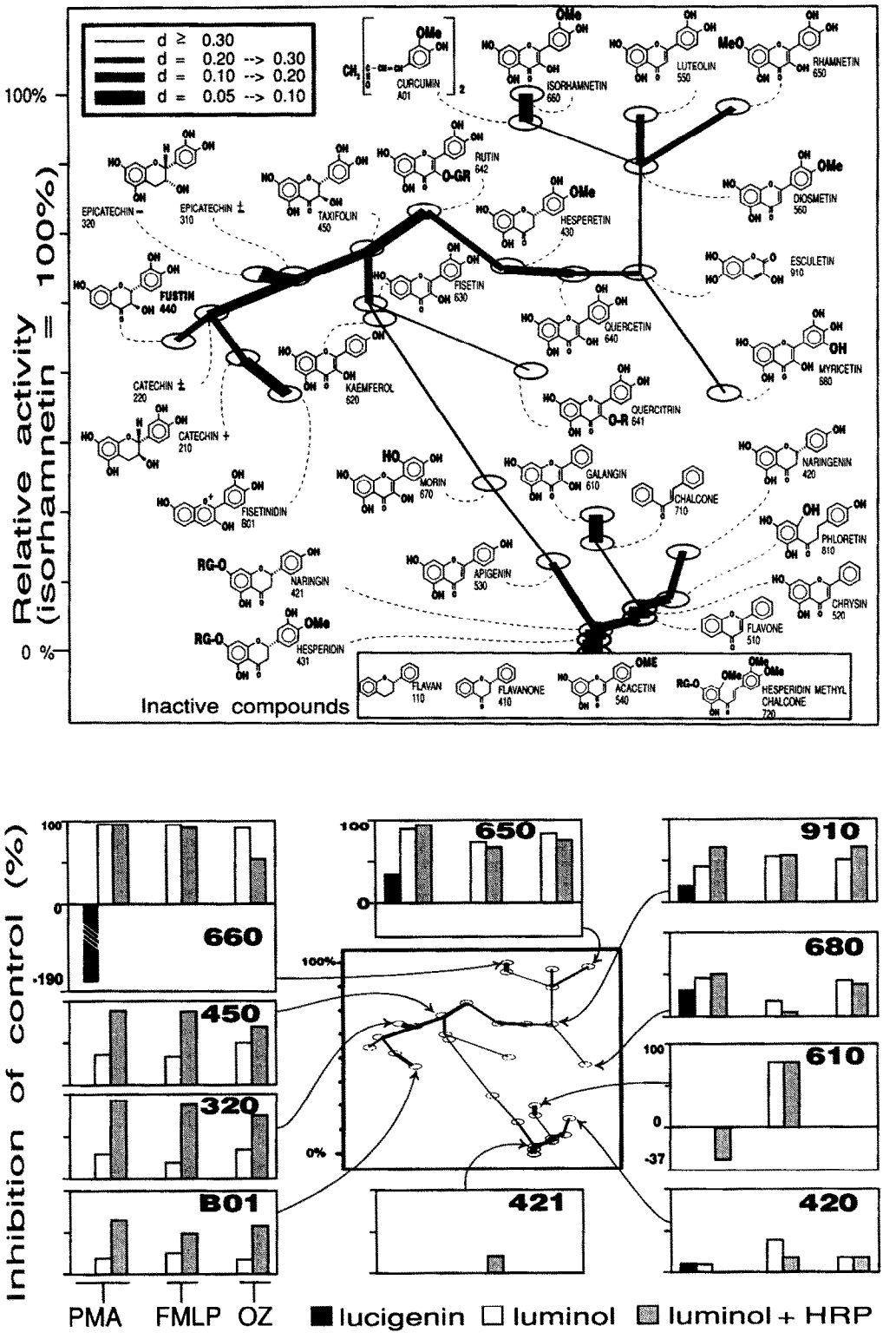



Fig. 4. MST analysis of the $10^{-5}$ M data in Table 1 after splitting into experimental values and antivalues in order to take into account the amplitude of the response as explained in the Materials and Methods. Affiliation between molecules (expressed according to an arbitrary scale) is indicated by the thickness of the connecting segments. Activity (ordinate) is expressed as the mean activity in all tests in comparison to isorhamnetin (100%). The individual activity profiles of molecules situated at branch ends or in key intermediate positions are shown in relation to a miniature version of the tree.

hydroxylation of kaempferol (**620**) to give morin (**670**) and the 5'-hydroxylation of quercetin (**640**) to give myricetin (**680**).

*Potency of flavonoids, ring A.* 7-Methoxylation of quercetin (**640**) to give rhamnetin (**650**) increases activity whereas glycosylation in position 7 (*o*-rutinose) markedly decreases activity [compare naringin (**421**) and naringenin (**420**) and also hesperidin (**431**) and hesperetin (**430**)]. A 5-hydroxy group explains the increase in the activity of taxifolin (**450**) over that of fustin (**440**) and the very slight increase in the activity of quercetin (**640**) over that of fisetin (**630**).

## DISCUSSION

With a few exceptions [e.g. Refs 28, 49], published SAR studies on flavonoids have all dealt with a single or at most two biological end-points in which one or more oxyradicals may intervene. We therefore considered it of particular interest to establish whether flavonoids with difference structural determinants are preferential scavengers of certain oxyradicals rather than others and whether their activity depends upon the signal pathway or mechanism leading to NADPH-oxidase activation and to subsequent steps.

In order to address this complex issue, we chose in the present study to investigate three types of variable simultaneously; compounds, activators and probes. The 34 test-compounds were for the most part natural flavonoids commonly used in medicinal preparations as well as a few derivatives and related chemical structures. NADPH-oxidase was activated by stimulating PMNs with three different agents (FMLP, PMA and OZ). Heterogeneity of oxyradical production was measured by chemiluminescence using two probes (luc and lum) and two experimental conditions (with or without HRP).

The only approach to the analysis of a data matrix such as the one generated in this study that is statistically valid is a multivariate approach that distinguishes correlation levels among all variables (from the strongest to the weakest), thus bringing to the fore those correlations that account for most or the variance (information content) of the system and relegating to a position of lesser importance those correlations that account for a lesser proportion of this variance. Such a multivariate approach has distinct advantages in that it extracts the information that is the most sound in spite of the imperfections of the experimental system or the inclusion of atypical molecules within the selected test-compound population, that it eliminates background noise and that, in the case of correspondence factorial analysis, it reduces to a minimum the number of graphic outputs required to describe the essence of the system without distortion.

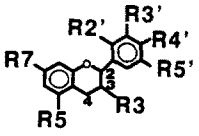
CFA first of all established that, for this population of molecules, the most important correlation that accounted for the largest proportion of the variance ($\varphi_1$ axis) was the opposition between inhibition of FMLP-stimulated PMNs and inhibition of PMA- or OZ-stimulated PMNs (Fig. 2). This result implies a fundamental difference in the manner in which this inhibition occurs and, conversely, a difference in the nature of the stimulatory action of these agents. Closer inspection (Fig. 3) revealed that the origin of this dichotomy resided in the specific inhibitory action of molecules lacking hydroxy groups on ring B (chrysin, chalcone, flavone and galangin), of ring B 4'-mono-hydroxylated molecules and of molecules that are glycosylated in position 7 (hesperidin and naringin). These compounds were specific inhibitors of FMLP activation measured by lum suggesting that, unlike the more hydrophilic multi-hydroxylated molecules, they might interfere with the lipid environment of the membrane FMLP receptor or with the receptor itself. An interaction with a membrane receptor has already been demonstrated for chrysin and chalcone which have affinity for the benzodiazepine (GABA) receptor [50]. Due to this specific interference with FMLP action, they might be usefully considered as lead compounds for the design of drugs for the early treatment of septic shock and of the respiratory distress syndrome.

An alternative explanation for this dichotomy could be sought at a later stage in the cascade of events induced by the bacterial peptide FMLP and one involving PKC. Most of the molecules that are not specific inhibitors of the FMLP-induced response are characterized by the presence of at least four hydroxy groups. Although low concentrations of quercetin have been found to stimulate PKC activity [51], hydroxylated flavonoids, at higher concentrations than those used in this study, inhibit PKC activity by competition with ATP for the catalytic site and, additionally, by a non-competitive inhibition with the histone substrate (regulatory domain) [10]. They also inhibit the action of several tyrosine protein kinases by competition with ATP, the inhibitory potency depending upon the number of hydroxy residues on the flavone rings [52–54]. This behavior can be compared to that of another chemical class of compounds, i.e. the hydroxylated di- and triphenylethylene derivatives, that can interfere with the catalytic and/or regulatory sites of PKC depending upon the number, nature and position of their hydroxyl groups [39]. It is therefore interesting to note that in the first factorial map, it is the compound with the most hydroxy groups, myricetin, that is the most discriminatory.

A recent study on the PKC inhibitor staurosporine concluded that PMA-inducible PKC may not be responsible for the regulation of early responses of the PLC pathway in FMLP-challenged neutrophils [55]. If the hydroxylated compounds are indeed PKC inhibitors in whole cells, the dichotomy we observe between FMLP- and PMA-induced stimulation may constitute indirect proof for these authors' hypothesis.

After the striking opposition between inhibition of neutrophil stimulation by FMLP, on the one hand, and PMA or OZ, on the other, the second feature that characterized an important fraction of the information content of the system ($\varphi_2$ axis) was the difference in inhibition observed in the presence or absence of HRP (Fig. 2). This difference is accounted for by the difference in inhibition by the catechins and by flavone and flavonol derivatives (Fig. 3). In the presence of HRP, lum measures the $H_2O_2$ formed principally by the action of SOD on

Table 3. Two alternative sets of structural features of flavonoids that are most characteristically associated with each of the experimental conditions




| Variable | 2′ | 3′ | 4′ | 5′ | 3 | 5 | 7 | $C_2C_3$ | $C_4$ |
|---|---|---|---|---|---|---|---|---|---|
| FMLP | H | H | H/(OH) | H | H/(OH) | ? | X | ? | ? |
| | X | X | X | X | X | ? | OGR | ? | ? |
| Lum + HRP | H | OH/(H) | OH/(OMe) | H | OH/(H) | OH/(H) | X | Unsaturated/ (saturated) | $H_2$/(O) |
| | X | X | X | X | X | X | OGR | X | O |
| Luc | H/(OH) | OH/(H) | OH/(OMe) | H/(OH) | OH/(OR) | OH | OH | Saturated | ? |
| | H | H | OH | H | H | OH | OH | Unsaturated | ? |
| Lum (alone) | Any substituents on ring B except for 3′,4′-OH | | | | OH/(H) | OH/(H) | OH/(H) | Unsaturated/ (saturated) | $H_2$/(O) |

Characters in parentheses are less favorable substituents, those outside are favorable.
X, any substituent of this study.
?, unknown.

the superoxide anion generated by NADPH-oxidase. Lum alone measures in addition a whole gamut of other ROS. Molecules such as catechins leading to low levels of radicals detected by lum plus HRP could thus reasonably be expected to be effective scavengers of $H_2O_2$, whereas those that are at a distance along the $\varphi_2$ axis might be less effective scavengers and also more effective inhibitors of MPO [13]. Although quercetin is able to scavenge hypochlorous acid, a species generated by the MPO/$H_2O_2$/$Cl^-$ system, it is an effective inhibitor of MPO, more effective than rutin [56].

The close proximity between stimulation by PMA and by OZ (Fig. 2), which implies great similarity in their reactivity to this population of molecules, cannot be interpreted with ease. Nevertheless, it is important to observe that these poles bring together molecules whose chemical structures are compatible with ROS destruction (scavenging activity) as opposed to molecules that might be more adapted to an interaction with the FMLP receptor.

For a detailed analysis of SAR, we classified the compounds in a MST that took into account the amplitude as well as the specificity of chemiluminescence inhibition. An advantage of an MST is that important information can be derived from the mere position of a molecule: if it is in a cluster, it is similar to others; if it is along a branch, it has an intermediate profile; it if is an isolated position at the end of a twig, it may be a new lead compound. The MST of our data classified the active compounds into three main families (Fig. 4).

The tree took root in the inactive flavonoids (flavane, flavanone, acacetin and hesperidin methyl chalcone), the only compounds devoid of a hydroxy group on ring B. The first compounds with a modicum of activity and that constituted the base of the trunk were hesperidin and naringin which, although mono-hydroxylated on ring B, are glycosylated at position 7 of ring A. At this point, there was an important offshoot leading to a family of compounds of low mean activity that included the non-flavonoids chalcone and phloretin whose profiles are virtually indistinguishable from those of the flavonoids flavone, chrysin, naringenin and galangin. Compared to compounds in higher branches, these derivatives were all unsubstituted or mono-hydroxylated on ring B, displayed proportionally lower mean activity on PMA-induced PMN stimulation and higher activity on FMLP-stimulation.

The main trunk of the tree was formed by four compounds of increasing activity: apigenin, morin, kaempferol and fisetin, all of which were $C_2$–$C_3$ unsaturated, hydroxylated at C7 and C4, and all of which had at least one (generally at C5), if not two (at C2′, 3′ or 3) extra hydroxy groups. This trunk led to a major intersection represented by taxifolin which combines five of the aforementioned hydroxy groups (C2′,3′,3,5,7) but which has a saturated C ring. To the left of taxifolin are the catechins with somewhat decreased overall mean activity but characterized, like taxifolin, by their pronounced inhibition of PMN stimulation when measured in the presence of HRP. To the right of taxifolin are equipotent compounds that lose their specificity versus lum minus HRP (i.e. rutin, hesperetin, quercetin and esculetin) and also more active compounds such as diosmetin and its three dissimilar offshoots: isorhamnetin with its non-flavonoid analog curcumin which both stimulate PMA-induced lucigenin luminescence, luteolin and rhamnetin. Luteolin with its C2′,3′,5,7 hydroxylated structure can be considered the chemical parent of these flavonoids which are each distinguished by the methoxylation of a different hydroxyl group with consequently different effects on specificity but little overall effect on mean potency.

These broad conclusions on SAR have been summarized in Table 3 which emphasizes the general compound characteristics associated with each

discriminatory biological variable in the order of their importance in accounting for the total variance of the system (FMLP, lum *plus* HRP, luc, lum alone). This is not a hard-and-fast mathematical classification but an assessment of the more favorable versus less favorable substituents (characters in parentheses) with respect to each of the biological variables. Compounds with the following ring B substituents proved to be particularly potent: 3′,4′-dihydroxy (luteolin, rhamnetin), 3′-methoxy, 4′-hydroxy (isorhamnetin) and 3′-hydroxy, 4′-methoxy (diosmetin). They could constitute valuable tools for further SAR studies on simpler molecules with the same chemical discriminants, such as for instance certain metabolites that might account for the biological activity of orally administered flavonoids [57], and lead to the design of novel drugs or prodrugs of simpler structure.

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