

Terpenoids: Sources, Structure Elucidation and Therapeutic Potential in Inflammation

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Abstract: Natural products research has lately undergone exponential growth owing to advances in isolation techniques and in synthetic methods design, as well as for the identification of a wide range of biological properties exhibited by these compounds. In the present review, general remarks on the chemical features, biosynthetic pathways, isolation and structure elucidation of terpenoids are briefly discussed. In addition to this, recent work done on anti-inflammatory terpenoids (diterpenoids, triterpenoids and sesquiterpene lactones) with special emphasis on the last new molecular targets evaluated is presented.

1. INTRODUCTION

Natural products chemistry has lately undergone explosive growth owing to advances in isolation techniques, synthetic methods, physico-chemical measurements, and new concepts, as well as for the wide range of biological properties exhibited by the substances biosynthesized by living organisms. A recent review pointed out that approximately 60% of the anti-tumour and anti-infective agents commercially available, or in late stages of clinical trials, are of natural product origin [1].

Terpenoids are the largest and most widespread class of secondary metabolites, mainly in plants and lower invertebrates. A few of them have been used for therapeutic purposes for centuries; but in recent decades the level of research activity in isolating and studying new substances has shown no sign of abating. The wide variety of biological activities shown by terpenoids, our knowledge of which is being continually augmented as new molecules are investigated, underlines their practical importance as a source of pharmacologically interesting agents.

2. TERPENOID: CHEMICAL REMARKS AND BIOSYNTHESIS

The tremendous biosynthetic potentialities of living organisms, and particularly plants, are not better illustrated than by the group of secondary metabolites known as

terpenoids. In general, the term terpene is used to denote compounds containing an integral number of C₅ units and chemically all terpenoids can be considered to be derived from the basic branched C₅ unit isoprene (2-methyl-1,3-butadiene, **1**) Fig. (1). According to the number of such C₅ units present in the molecule, terpenoids are classified into hemi-, mono-, sesqui-, di-, sester-, tri-, and tetraterpenoids (carotenoids), having 1, 2, 3, 4, 5, 6, and 8 isoprenoid C₅ residues, respectively.

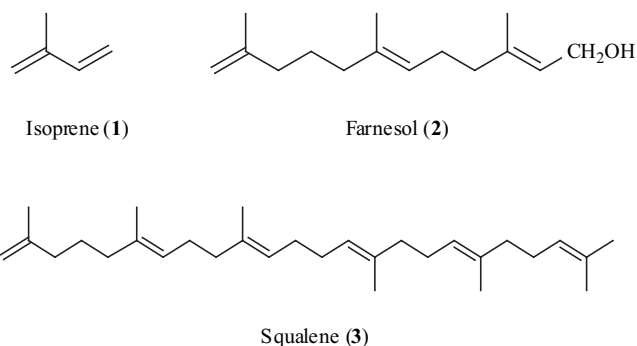


Fig. (1). Some acyclic terpenoids.

Sometimes the isoprenoid residue is indicated by *ip*. Thus farnesol (**2**, a sesquiterpene, Fig. 1) is *ip ip ip*, indicating that it is formally made up of three *ip* residues joined head-to-tail. Note that the branched end of the isoprene unit is regarded as the 'head' and the unbranched end as the 'tail'. Although the head-to-tail junction is the most common method of linking isoprene units together in mono-, sesqui-, di-, and sesterterpenoids, tail-to-tail junctions also occur and are indicated by *ip pi*. This type of junction is to be present at the centre of tri- and tetraterpene molecules; squalene, for example (**3**, Fig. 1), is formally

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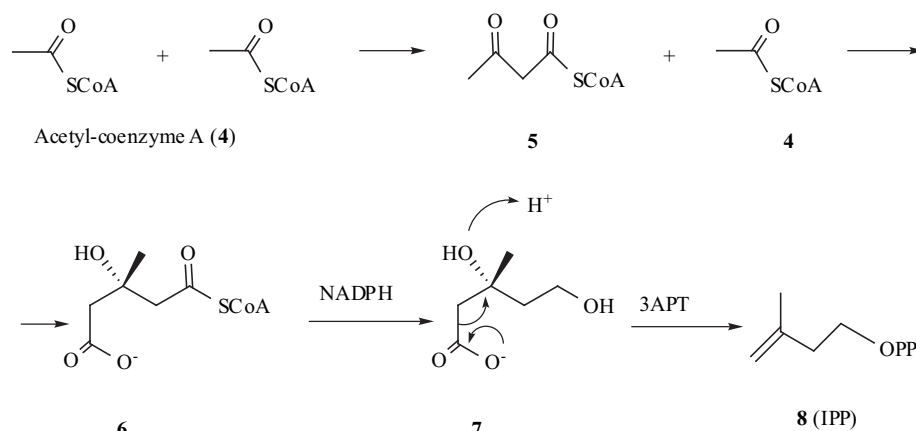


Fig. (2). Mevalonate pathway for isopentenyl diphosphate (IPP) biosynthesis.

composed of two farnesyl residues joined tail-to-tail and it is represented by *ip ip ip pi pi pi*.

Apart from the terpenes cited above, there are natural polyisoprenoid substances such as rubber, gutta, and chicle, produced by many plants, and a great number of naturally occurring compounds in which a terpenoid structural part is frequently found combined with non-terpenoid moieties.

The immense variety of structural types and compounds classified as terpenoids is firstly due to the number of isoprene units integrating their molecules that originates different linear arrangements. These intermediates are biosynthetically modified by various cyclizations and rearrangements of the hydrocarbon skeleton. Extensive biosynthetic modifications in the chemical functionality of these precursors lead to compounds with complex structures possessing several stereogenic centres.

The rapidly growing knowledge of terpenoid structures led naturally to questions concerning the transformations and functions of these biological molecules and hence to the interest in their biosynthesis.

The formation of the common isoprene-derived subunit of the terpenoids has been extensively studied over the last 50 years, leading to a generally accepted pathway (Fig. 2) from acetate activated as acetyl-coenzyme A (4), via acetoacetyl-coenzyme A (5), 3-hydroxy-3-methylglutaryl-coenzyme A (6), and mevalonate (7, MVA) to isopentenyl diphosphate (8, IPP), the first precursor possessing the branched C₅ isoprene skeleton [2].

Although the mevalonate biosynthetic pathway shown in Fig. (2) had been considered for many years as the unique route for the synthesis of IPP (8) in living organisms, recent studies have demonstrated that IPP can also be

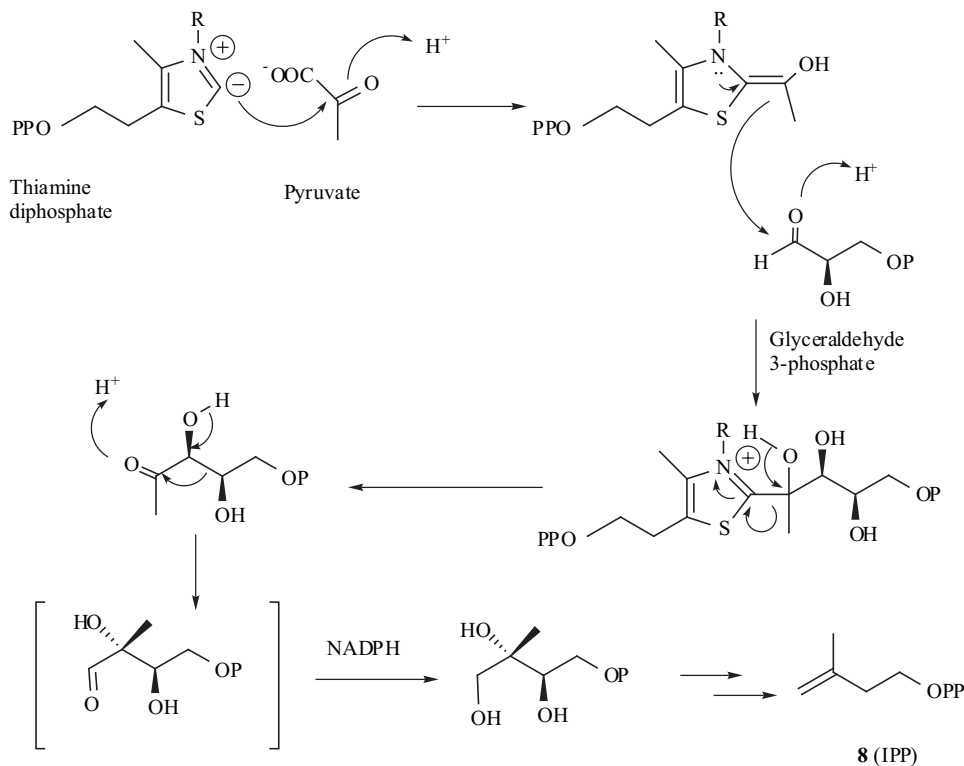


Fig. (3). Glyceraldehyde 3-phosphate/pyruvate pathway for isopentenyl diphosphate (IPP) biosynthesis.

biosynthesized by a mevalonate-independent pathway in bacteria, algae, and higher plants [2], as it is summarized in Fig. (3). These studies showed that glyceraldehyde 3-phosphate (or eventually glyceraldehyde) and a C₂ unit derived from pyruvate decarboxylation are the only precursors of the C₅ skeleton of IPP (8, Fig. 3) for isoprenoid biosynthesis in bacteria like *Escherichia coli* and *Zymomonas* [3], as well as for mono-, sesqui-, and diterpenes in higher plants [4-5] and other living organisms [6]. In some cases, however, other studies [7] have evidenced that IPP is synthesized, even in the same organism, by both mevalonate and triose 3-phosphate/pyruvate pathways [Fig. (2) and (3), respectively], depending on the intracellular site of the living organism where the biosynthesis of each type of terpenoid is localized [4,7].

Isopentenyl diphosphate (8, IPP) is converted into all the different terpenes and terpenoids found in nature as it is shown in Fig. (4). IPP (8) is firstly isomerized by the sulphhydryl enzyme (IPP isomerase) to dimethylallyl diphosphate (9, DMAPP, Fig. 4). Either of these compounds (IPP and DMAPP) can be transformed into hemiterpenoids (C₅) (Fig. 4).

DMAPP also acts as a starter for chain elongation and, under the influence of the enzyme prenyltransferase, condenses with a molecule of IPP to form the C₁₀ derivative geranyl diphosphate (10, GPP, a monoterpene). The GPP (10) can then either be channelled into monoterpene biosynthesis or condense with another IPP molecule to form the C₁₅ compound farnesyl diphosphate (11, FPP, a

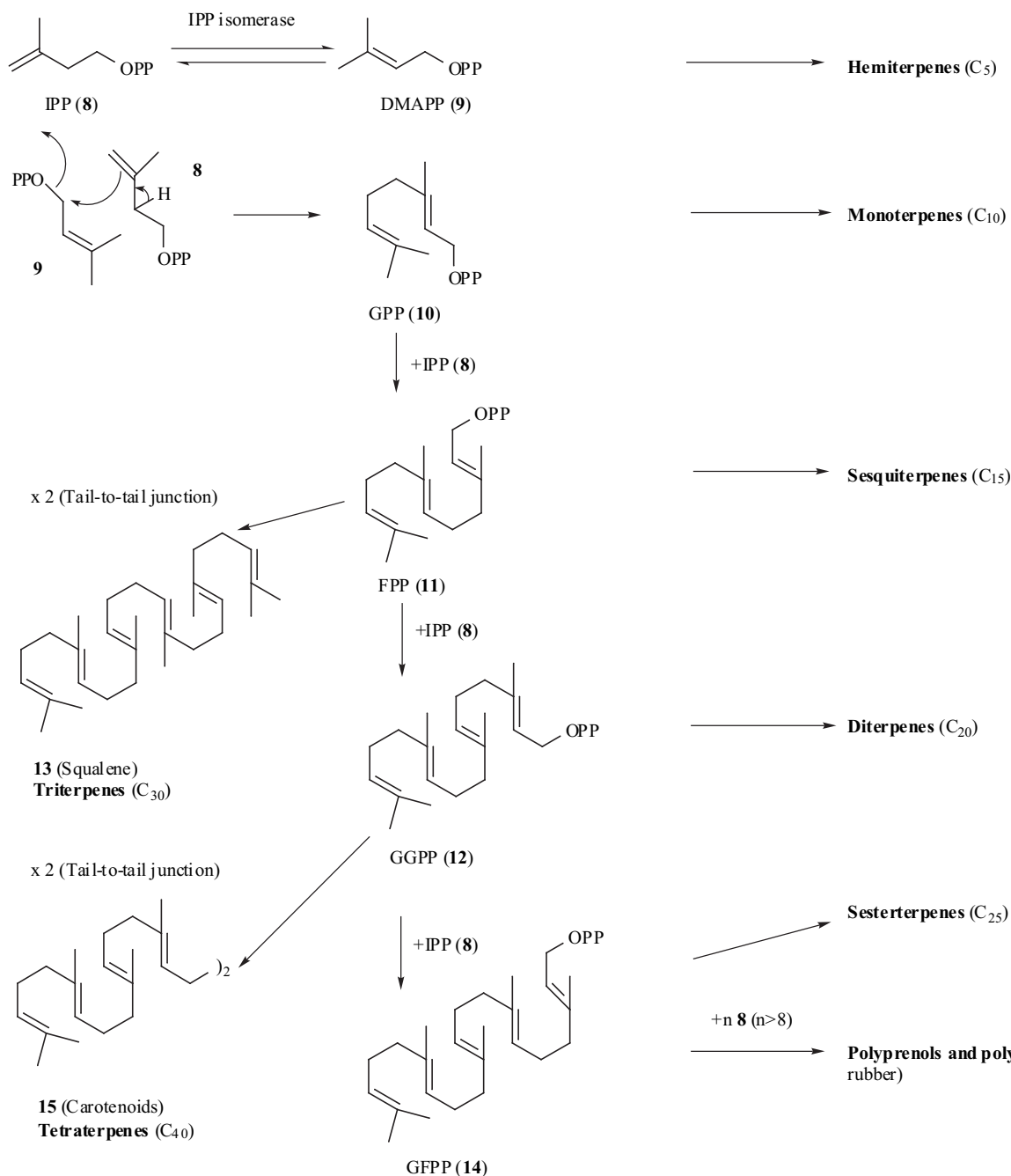


Fig. (4). General scheme of terpenoid biosynthesis.

sesquiterpenoid), which has three possible metabolic fates, to form sesquiterpenes, to undergo chain extension to form the C₂₀ compound geranylgeranyl diphosphate (**12**, GGPP, a diterpene), or undergo a tail-to-tail dimerization with the formation of the C₃₀ compounds (**13**, triterpene). GGPP (**12**) has the same type of metabolic options as FPP (**11**), generation of diterpenes (C₂₀), chain elongation to the C₂₅ compound (geranylgeranyl diphosphate **14**, GFPP), and tail-to-tail dimerization to tetraterpenes (**15**, C₄₀ compounds, carotenoids). GFPP (**14**) generally undergoes further multiple chain extension to yield polyprenyl diphosphates and ultimately polyprenols and polyterpenes (rubber, gutta, chicle) and only occasionally it is transformed into sesterterpenes (C₂₅ compounds).

3. EXTRACTION AND ISOLATION

Terpenoids generally occur as complex mixtures, which can be extracted from the natural source by a great variety of methods. The essential oils, which are constituted by mixtures of lower terpenes (mono- and sesquiterpenes), are extracted subjecting plants to steam distillation, but in general the extraction of terpenoids from plant materials is carried out by successive solvent extraction (with increasing polarity of the solvents) or by liquid/liquid partition from a first acetonic or alcoholic extract. Plants are generally dried, ground and then extracted in a Soxhlet or, more convenient, left to macerate at room temperature. Care must be taken during the extraction, and also during the isolation process, to avoid the occurrence of artefacts.

Purified terpenoids are required for complete spectroscopic identification and full characterization of new compounds, for biological testing, and for the supply of pharmaceuticals, standards, and starting materials for synthetic work. Obtaining pure products from an extract can be a very long, tedious and expensive undertaking, involving many steps. Sometimes only minute amounts of the desired compounds are at hand. Thus, it is an advantage to have access to as many different methods as possible in order to aid the isolation process. Different chromatographic techniques, such as column chromatography, preparative HPLC, radial chromatography, etc. [8] are often found to be adequate for isolation of the pure individual constituents in adequate amounts. However, complex mixtures of structurally very close compounds, such as essential oils, require special techniques (e.g. preparative HPLC- and GC-mass spectrometry) for isolating and identifying each substance.

4. STRUCTURE ELUCIDATION

Structures of the isolated pure terpenoids are generally investigated by a combination of chemical and spectroscopic methods. However, the current trend is to determine structures by spectroscopic methods alone, which have the advantage of allowing the examination of a small amount of the intact terpene prior to any treatment, which might produce artefacts. With the availability of powerful modern nuclear magnetic resonance (NMR) techniques, the structural

elucidation of a new terpenoid has in many cases become routine, and the identification of a substance with a previously known compound is also very easy, taking into account the enormous literature data on these metabolites that have been generated during the past decades.

In general, a careful study of the ¹H and ¹³C NMR spectra of a terpenoid, together with the data provided by other 2D NMR experiments (e.g. COSY, TOCSY, NOESY, HSQC, HMBC, etc), allows the elucidation of its structure, including the relative stereochemistry of chiral centres. Moreover, if the compound studied is a crystalline solid, an X-ray diffraction analysis can clear up its structure.

Terpenoids possessing one or more chiral centres, except for triterpenoids, can be biosynthesized in both enantiomeric forms by living organisms. Thus, the determination of the absolute configuration of a terpene is crucial for understanding its role in biological processes. The absolute stereochemistry of terpenoids can be established by several procedures which include X-ray analysis (specially for molecules possessing a heavy atom), chiroptical data (optical rotatory dispersion, circular dichroism, exciton chirality, etc), [9-10], chemical correlation with a compound of known absolute configuration, resolution of racemates, Mosher's and related NMR methods as well as enzymatic reactions [11].

5. ANTI-INFLAMMATORY ACTIVITY OF SELECTED TERPENOIDS

This section of the review gives an account of the work done on bioactive terpenoids from plants, with regard to their anti-inflammatory properties. After considering some general aspects of the inflammation, with special emphasis on the new targets evaluated in the search for new potential drugs, the anti-inflammatory activities of diterpenoids, triterpenoids and sesquiterpene lactones reported during the last five years have been reviewed.

5.1 General Remarks on Inflammation

Inflammation is a complex pathophysiological process characterised by redness, heat, swelling and organ decreased function. It is mediated by a variety of signalling molecules produced by neutrophils, monocyte/macrophages, mast cells, platelets and lymphocytes, as well as by the activation of the complement cascade which bring about oedema formation as a result of extravasation of fluid and proteins and accumulation of leukocytes at the inflammation site.

Macrophages play a crucial role in modulating the initiation and perpetuation of the inflammatory response. Activation of these cells with proinflammatory cytokines and bacterial cell components promotes the synthesis and release of large amounts of nitric oxide, eicosanoids and bioactive lipids, mediators involved in the inflammatory onset, as well as cytokines (tumor-necrosis factor (TNF- α) and interleukine-1 (IL-1 β)). The oxidative metabolism of arachidonic acid (AA) leads to the synthesis of prostaglandins (PGs) and leukotrienes (LTs).

Cyclooxygenases catalyse the first rate-limiting step in the synthesis of prostaglandins and thromboxanes (TXs) from arachidonic acid by the two cyclooxygenases identified: the constitutive cyclooxygenase-1 (COX-1) and the inducible cyclooxygenase-2 (COX-2) [12]. Nitric oxide (NO) and prostaglandin E₂ (PGE₂) are critical mediators produced by two inducible enzymes, nitric oxide synthase (iNOS or NOS-2) and COX-2, respectively and are thought to be responsible for the massive production of NO and prostaglandins, which have been implicated in the tissue destruction and pathogenesis of a number of immunological and inflammatory diseases, including septic shock, rheumatoid arthritis, diabetes, among others.

NF- κ B comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response [13], promoting the expression of specific cellular genes involved in host defense such as pro-inflammatory cytokines, chemokines, cell adhesion molecules and various matrix metalloproteinases. This transcription factor promotes the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, including NOS-2, which generates NO and the inducible COX-2, which generates prostanoids [14]. NF- κ B is present constitutively in the cytosol of cells, where it is retained through the interaction with inhibitory I κ B proteins that mask the nuclear localization domain of the complex (Fig. 5). NF- κ B-dependent gene transcription requires the phosphorylation of I κ B α by IKK2, which

releases this inhibitory component from the dimmer of Rel proteins (mainly p50, p65 and c-Rel) and is followed by ubiquitination and degradation of the phospho-I κ B by the proteasome [15]. Biochemical, pharmacological and genetic data indicate that the control of NF- κ B activation constitutes a relevant target for the treatment of several inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. For this reason, the research of molecules endowed with the ability to inhibit the consecutive steps leading to NF- κ B activation has been a subject of current interest. In this regard, among the natural products assayed, various terpenoids have been described as potent inhibitors of NF- κ B activation in response to proinflammatory stimulation.

In the literature, several *in vivo* and *ex vivo* bioassays have been used to evaluate potential anti-inflammatory agents.

Ear oedema, paw oedema and air pouch models of acute inflammation have been extensively used as *in vivo* animal models. Ear oedema can be induced by a great diversity of irritant agents (e.g. phorbol ester TPA, AA and croton oil) and different inflammatory mechanisms have been suggested [16]. Inflammation induced by TPA is related to the activation of phospholipase A₂ (PLA₂), which releases AA from the cell membrane and is metabolized to PGs and LTs. Myeloperoxidase (MPO) activity, a hemoprotein located in the azurophil granules of neutrophils, used as biochemical

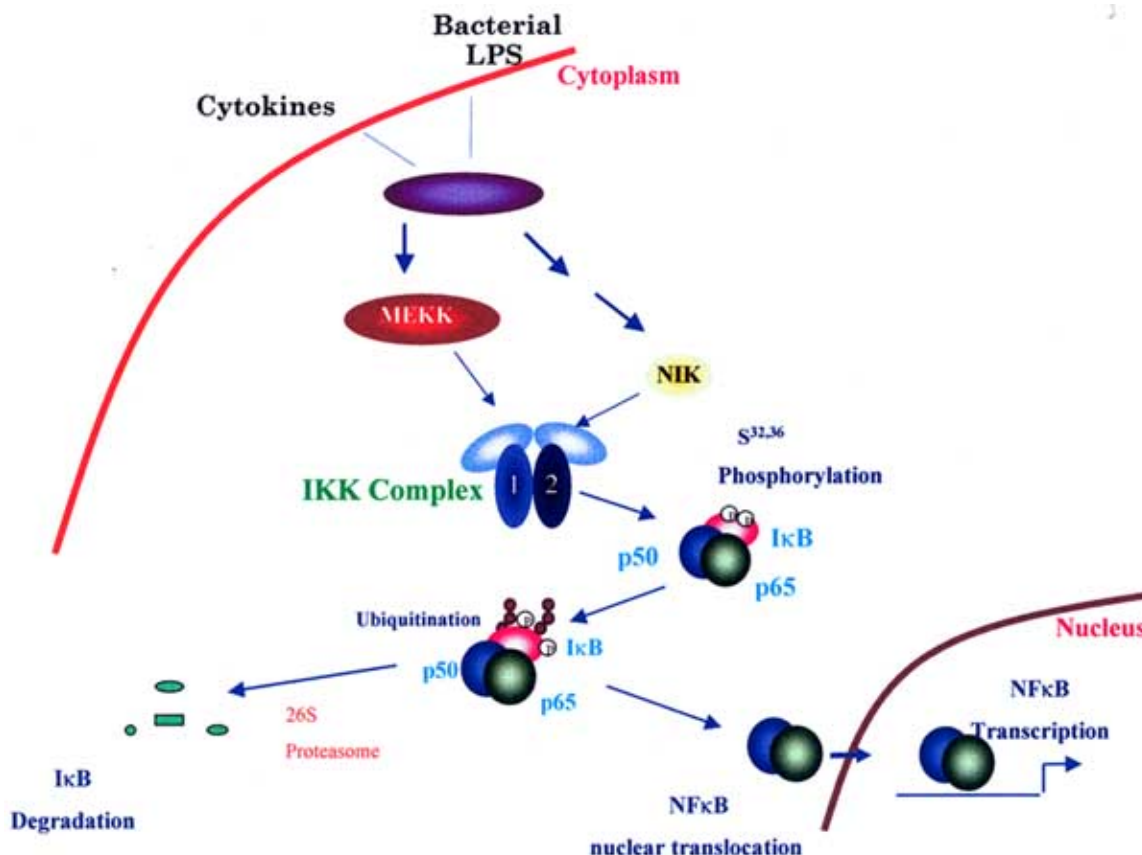


Fig. (5). Signalling pathways leading to NF- κ B activation. (MEKK: MAP kinase kinase).

marker for leukocyte infiltration can also be measured in this model. Substances that inhibit this kind of oedema could be inhibitors of COX and/or 5-lipoxygenase (5-LOX). Indeed, AA-induced oedema has been reported as a model responsive for 5-LO inhibitors. Different agents (*e.g.* carrageenan and serotonin) can be elicitors of acute paw oedema inflammation in various species (rat, mice). Finally, air pouch model induced by carrageenan or zymosan produces an inflammatory exudate, which can be collected some hours after induction to measure eicosanoid synthesis and other inflammatory mediators [17].

Macrophages play a crucial role in modulating the initiation and perpetuation of the inflammatory response. Activation of these cells causes the release of eicosanoids, NO and pro-inflammatory cytokines, including TNF- α and IL-1 β [18]. Cytokines, as well as bacterial LPS, induce the expression of enzymes such as COX-2, NOS-2 and matrix metalloproteinases in macrophages and other cells, which results in the release of inflammatory mediators and tissue remodelling. This cell system has been widely used to evaluate agents able to block inflammatory mediator production. NF- κ B, central mediator of the immune response, regulates the transcription of various pro-inflammatory cytokines and genes encoding for COX-2, NOS-2 and cell adhesion molecules among other proteins. Moreover, these cells can also be activated by different stimulus, allowing a precise definition of signalling pathways that are inhibited in response to anti-inflammatory molecules, including terpenoids. This *ex vivo* methodology offers new strategies to underscore intracellular targets for these molecules.

5.2 Sesquiterpene Lactones

The sesquiterpene lactones are natural products widely distributed in the Asteraceae and are known for their wide variety of biological activities [19-22]. Some medicinal plants owe their anti-inflammatory properties to their content on sesquiterpene lactones.

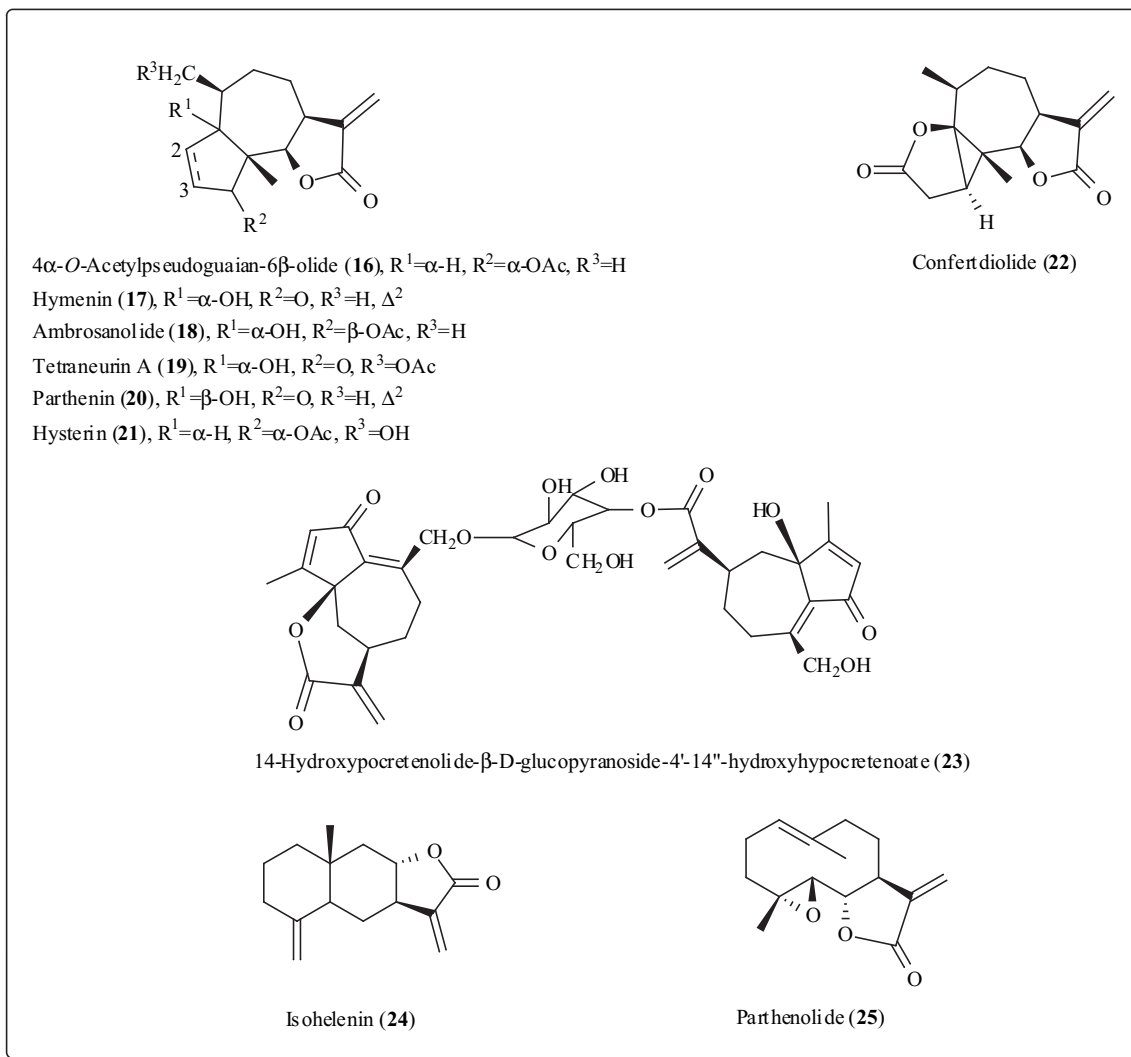
In a general screening using the model of oedema induced by carrageenan, Hall *et al.* [22] observed *in vivo* that the α -methylene- γ -lactone moiety was required for activity of sesquiterpene lactones. When the 11,13-methylene group was saturated or masked, significant activity was lost. These structures are considered to be involved in the mechanism of action of sesquiterpene lactones through alkylation of biological nucleophiles by a Michael-type addition reaction. The covalent binding to free sulphhydryl groups in proteins can inhibit a large number of biological processes such as neutrophil migration, lysosomal rupture and different enzymatic activities. However, the simple α -methylene- γ -lactones caused minimal anti-inflammatory activity, which means that for pharmacological activity other steric requisites must be fulfilled. In fact, the complete structure of the sesquiterpene lactone appears to be important for the activity, and the derivatives of pseudoguaianolide and germacranolide skeletal types are the most active. Recently, it has been reported that differences in molecular conformation as well as lipophilicity may affect the steric accessibility of Michael addition sites [23].

There are a few studies reporting the *in vivo* activity of this type of compounds. Recio *et al.* have reported the *in vivo* effect on acute and chronic inflammation of seven pseudoguaianolide type of sesquiterpene lactones: 4- α -O-acetyl-pseudoguaian-6 β -olide (**16**), hymenin (**17**), ambrosanolide (**18**), tetraeurin (**19**), parthenin (**20**), hysterin (**21**) and confertdiolide (**22**) isolated from several species of *Parthenium* [23]. All the compounds showed activity against TPA-induced mouse ear oedema. Compounds **18**, **20** and **22** inhibited the EPP-induced oedema. However, none of them were active on AA-induced oedema. The only sesquiterpene lactone orally active against the paw mouse oedema induced by carrageenan was **22**, which gave a 46% oedema inhibition after 3 h. Confertdiolide (**22**) was the most active compound when assayed against the chronic inflammation induced by repeated application of TPA on mouse ear. It reduced the oedema by 87 % and had a moderate effect against leukocyte recruitment.

The anti-inflammatory activities of hypocretenolides, a small group of sesquiterpene lactones with an unusual ring structure isolated from the medicinal plant *Leontodon hispidus* (Asteraceae), have been evaluated [24]. Croton oil-induced mouse ear oedema was used to evaluate the *in vivo* anti-inflammatory activity. Furthermore, in order to gather first clues about possible molecular targets, which might be affected in inflammatory events, these compounds were evaluated for their ability to interfere with NO production and NF- κ B activation. Compound **23**, 14-hydroxyhypocretenolide- β -D-glucoside-4-14-hydroxyhypocretenoate, significantly exhibited *in vivo* anti-inflammatory activity. The authors concluded that polarity might be essential for inhibiting the activation of NF- κ B.

More recently, attention has been paid to the study of sesquiterpene lactones, which have been extensively tested as potential inhibitors of transcription factor NF- κ B. Bork *et al.* [25] firstly described the preventive effects of isohelenin (**24**) and parthenolide (**25**) on NF- κ B activation and identified the eudesmanolide and germacranolide type of sesquiterpene lactones as potent non-antioxidant inhibitors of NF- κ B. Hehner *et al.* [26] next investigated the structural features of the sesquiterpene lactones which confer inhibitory activity on NF- κ B activation pathways. Two structural hallmarks of these compounds are an isoprenoid ring system and a lactone ring. They concluded that isoprenoids that lacked either the lactone or the exomethylene group in the α -position of the lactone function displayed no inhibitory effect on the pathway leading to NF- κ B activation. Another interesting structural element especially described for parthenolide (**25**) is its epoxide ring that is also a likely site for the addition of nucleophilic reagents. This study showed that sesquiterpene lactones block a common step in NF- κ B. They did not interfere with the generation of oxygen radicals, but prevented the induced degradation of I κ B α and I κ B β .

In the last past years, several authors have evaluated the anti-inflammatory activity of sesquiterpene lactones possessing different skeletons (germacranolide, eudesmanolide, guaianolide) using the transcription factor NF- κ B as the molecular target [27-33]. Lactones possessing an α -methylene- γ -butyrolactone and a second reactive



grouping were the most active. It is thought that they inhibit NF- κ B by selectively alkylating the p65 subunit, probably by reacting with cysteine residues. In order to gain further insight into the structure-activity relationships and the molecular mechanism of action of these compounds, Koch *et al.* [34] have evaluated four different germacranolide sesquiterpene lactones for their effect on the production of proinflammatory cytokines (IL-1 β , IL-6 and TNF- α). It was concluded that compounds possessing an α -methylene- γ -lactone motif and a conjugated carbonyl group were active inhibitors of cytokine synthesis. The results obtained correlated well with the effect observed in the NF- κ B assay.

5.3 Diterpenoids

Diterpenoids are a large and ubiquitous family of isoprenoid products derived from 2E, 6E, 10E-geranylgeranyl pyrophosphate. Most of the diterpenoids found in recent years have been isolated from the Compositae and Lamiaceae families.

According to the chemical structure, the diterpenoids are classified in two main groups: acyclic and cyclic

compounds. On biogenetic considerations, the cyclic diterpenoids are classified into:

1. Bicyclic diterpenoids: labdanes and clerodanes.
2. Tricyclic diterpenoids: pimaranes, isopimaranes, abietanes, cassanes and rosanes.
3. Tetracyclic diterpenoids: kaurenes, atisirenes, beyerenes and gibberellins.
4. Pentacyclic diterpenoids: trachylobanes.
5. Macrocyclic diterpenoids: cembrenes, casbenes, taxanes..
6. Miscellaneous diterpenoids: briarane, vibsane..

Several biological actions have been reported for diterpenes including antibacterial, antifungal, anti-inflammatory, antileishmanial, antialgal, cytotoxic and anti-tumour activities [35-39]. Concerning the anti-inflammatory properties of diterpenes, we have previously reported that andalusol (26), a labdane diterpene isolated from *Sideritis foetens* (Lamiaceae) showed anti-inflammatory activity in *in*

vivo models of inflammation [16]. It also showed an inhibitory activity on NO production (IC_{50} : 10.5 μ M) in activated J774 macrophages through inhibition of macrophage expression of NOS-2 enzyme, an effect correlated with a marked inhibition of nuclear factor NF- κ B activation, a necessary transcription factor for NOS-2 expression in response to bacterial lipopolysaccharide (LPS) and interferon- γ (IFN- γ) [40].

Kaurene diterpenes have also been reported as NO inhibitors [41-42]. Four kaurene diterpenes were isolated from the genus *Isodon* (Lamiaceae) namely: kamebanin (**27**), kamebacetal A (**28**), kamebakaurin (**29**) and excisanin A (**30**). They significantly inhibited NO synthesis in activated RAW 264.7 cells, with IC_{50} values in the 100-500 nM range. They also inhibited PGE₂ production showing IC_{50} values in the 1-5 μ M range. Interestingly, the different IC_{50} ranges for NO and PGE₂ synthesis reflects the complex regulation of the expression of COX-2 and NOS-2 genes and, perhaps, the existence of multiple targets influenced by these kaurene diterpenes. These kaurene diterpenes exhibited a closed structure and contained an α -methylene-pentane moiety as a common functional group.

All diterpenes inhibited LPS-induced DNA binding activity of NF- κ B dose-dependently and NF- κ B activation was completely inhibited in the presence of 26.6 μ M of kamebakaunin (**29**) and excisanin A (**30**).

The results on kaurene diterpenes above described are in agreement with those reported by Castrillo *et al.* [42]. These authors showed inhibition of nuclear factor NF- κ B pathway by three kaurene diterpenes [foliol (**31**), linearol (**32**) and *ent*-kaur-16-en-19-oic acid (**33**)] also in macrophages. These compounds potently inhibited NO release through inhibition of the expression of NOS-2, but were less efficient regarding the effects on COX-2. Again, the apparent IC_{50} values for NO synthesis were 3-5 μ M whereas the diterpene linearol (**32**) exhibited an IC_{50} of 9 μ M.

To investigate the mechanism of action of kaurenes on macrophage function, the effects on NF- κ B activity were analyzed. Kaurene diterpenes inhibited the activation of NF- κ B through a mechanism that involved an impairment of IKK activity, by inhibiting NIK, a member of the MAPK kinase superfamily that interacts with tumor necrosis factor receptor-associated factors and mediate the activation of NF- κ B by these receptors.

Teucrin A (**34**), 19-acetylnaphalin (**35**) and eriocephalin (**36**), three clerodane diterpenes, were also evaluated by Castrillo *et al.* [42]. However, these clerodanes were unable to modify the inflammatory response to proinflammatory stimulation, thus being inactive.

Our group has also evaluated the effect of some diterpenes possessing different skeletons on the cyclooxygenase and 5-lipoxygenase pathways of the arachidonate metabolism and on NO production [18]. Two abietane diterpenes, aethiopinone (**37**) and 11, 12, dihydroxy-6-oxo-8, 11, 13-abietatriene (**38**) and the rosane lagascatriol (**39**) showed a remarkable effect on the COX-1 pathway of PGE₂ release. Only the two latter diterpenes

showed inhibition on the COX-2 pathway. In addition to this, all compounds assayed were inhibitors of LTC₄ release with $IC_{50} \leq 10 \mu$ M. Compound **38** significantly inhibited NO production.

Benrezzouk *et al.* [43] reported the inhibitory effect of two neo-clerodane diterpenes: E-isolaridial (**40**) and its methylketone (**41**) derivative isolated from *Linaria saxatilis* (Scrophulariaceae) on Phospholipase A₂ (PLA₂) and other enzyme activities involved in the inflammatory process. Both compounds inhibited secretory PLA₂ activity with IC_{50} values of 0.20 and 0.49 μ M, respectively. These diterpenes also decreased the cell-free 5-LO activity and A23187-induced neutrophil LTB₄ biosynthesis. In contrast, none of the compounds affected COX-1, COX-2 and NOS-2 activities in cell-free assays.

The anti-inflammatory activity of the abietic acid (**42**) isolated from *Pimenta racemosa* (Myrtaceae) has been tested *in vivo* and *in vitro* [44]. This compound significantly inhibited rat paw oedema induced by carrageenan in a time-dependent manner and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear oedema, after oral or topical administration. This diterpene also prevented the release of some inflammatory mediators *in vitro* as PGE₂, being less active on NO, TNF- α and IL-1 β production.

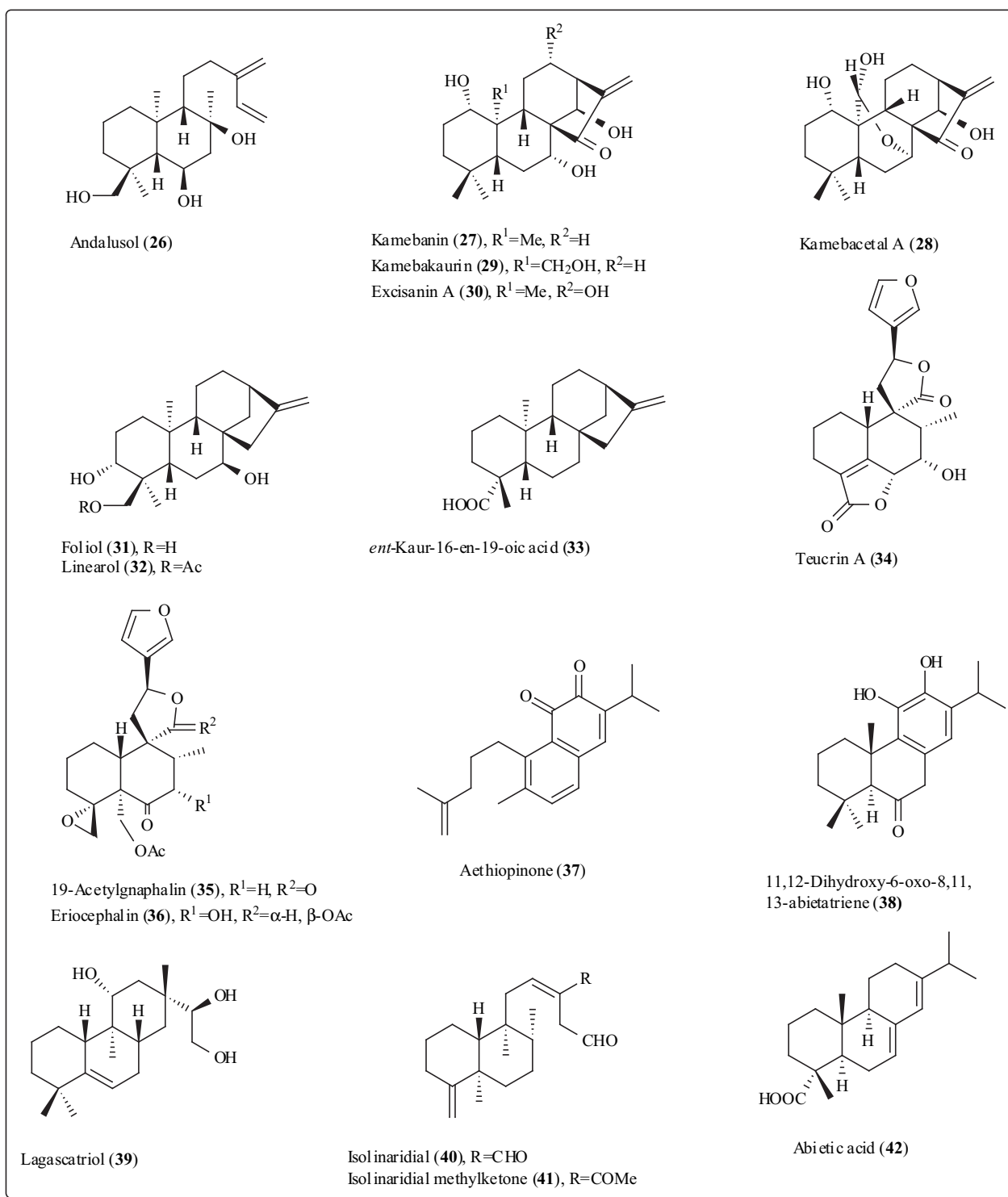
5.4 Triterpenoids

Naturally occurring triterpenoids often exhibit a variety of biological activities such as anti-inflammatory [45-46], anti-HIV [47], anti-tumour-promoting [48-49], ichthyotoxic [50] and antimycobacterial [19] activities.

Recent reviews classify triterpenoid according to chemical characteristics or biogenetic origin [51-52] but in the present review they will be classified only according to their anti-inflammatory properties. Most of the anti-inflammatory triterpenes isolated have oleanane, ursane, taraxastane, lupane and lanostane skeletons. The triterpenoids constitute a large diverse group derived from squalene, widespread in a wide range of plant families. Oleananes and ursanes often occur together and have been reported in Araliaceae, Asclepidaceae, Bignoniaceae, Cactaceae, Campanulaceae, Celastraceae, Compositae, Labiatae, Leguminosae and Urticaceae, among others. Lupanes are also widespread in the Apocynaceae, Celastraceae, Compositae, Euphorbiaceae, Labiatae and Rhamnaceae. Lanostanes, which occur widely in fungi and marine organisms, are also found in plants, *e.g.* in the Ramulaceae, Orchidaceae and Pinaceae. Friedelanes are more restricted in their distribution and are found mainly in the Celastraceae.

Rios *et al.* [53] have extensively revised natural triterpenoids as anti-inflammatory agents. The present paper adds information on new compounds isolated and published in the literature from 1997 to 2001.

Several triterpenoids have been recently tested on *in vivo* models of acute and chronic inflammation: TPA-induced ear oedema, arachidonic acid (AA)-induced ear oedema, ethyl



phenylpropiolate, protein kinase C activators, mezerein, two 12-deoxyphorbol-13-monoesters [13-tetradecanoate (DPT) and 13-phenylacetate (DPP)], bryostatin 1, resiniferatoxin and xylene, PLA₂-hind paw oedema and adjuvant arthritis.

Many kinds of pentacyclic triterpenes have been reported as anti-inflammatory agents [54-55], but only a restricted group of tetracyclic derivatives have been described and reviewed [56]. The presence of tetracyclic triterpenes in nature is less notorious than that of pentacyclic derivatives, and their occurrence is limited to a minor number of

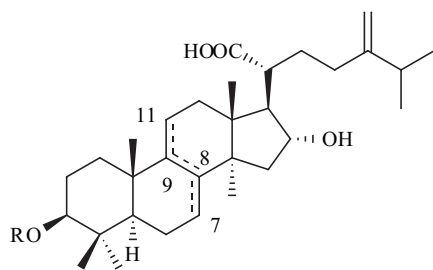
botanical families. Lanostanes and closely related compounds, for example cycloartanes and euphanes, are widely distributed in fungi and marine organisms, but many compounds have also been isolated from higher plants. Lanostanes are the most relevant group of the tetracyclic triterpenes and many compounds have been described as anti-inflammatory agents.

Pachymic (43) and dehydrotumulolic (44) acids isolated from the fungus *Poria cocos* (Polyporaceae) were studied in different models of inflammation [57]. They proved to be

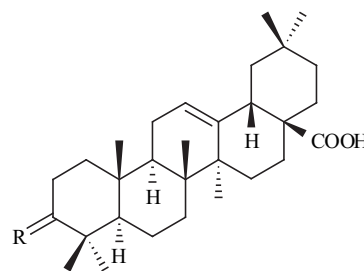
active in most of the assays tested. None of them were active against AA-induced ear oedema. Dehydrotumulosic acid (**44**) significantly diminished ear oedema induced by ethylphenylpropiolate (49 % oedema inhibition), while pachymic acid (**43**) was ineffective. When the putative corticoid-like mechanism of both compounds was explored, pachymic acid activity was partially abolished by the glucocorticoid receptor antagonist progesterone, but dehydrotumulosic acid activity was not affected, suggesting an anti-inflammatory activity independent of the glucocorticoid mechanism for this last lanostane triterpenoid. On the PLA₂-induced mouse paw oedema, both compounds were active, exhibiting inhibition percentages ca. 60 % [57].

Yasukawa *et al.* [58] reported the anti-inflammatory activity of the β -*p*-hydroxybenzoyldehydrotumulosic acid (**45**) from *Poria cocos*. This triterpene showed marked inhibitory activity against TPA and AA-ear oedema in mice (ID₅₀: 0.27 and 1.25 mg/ear in TPA and AA-induced oedema, respectively).

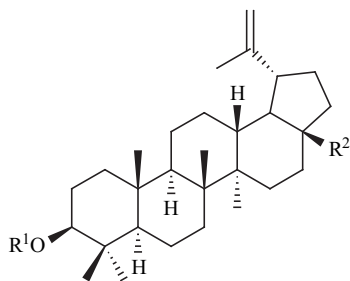
Oleananes constitute the largest and most important group of active triterpenes. Giner-Lanza *et al.* [59] have described the anti-inflammatory activity of both oleanolic (**46**) and oleanonic (**47**) acids in different inflammation tests. In the TPA-model of chronic inflammation induced by multiple applications, oleanonic acid (**47**) showed a significant effect with 45 % inhibition, in contrast to



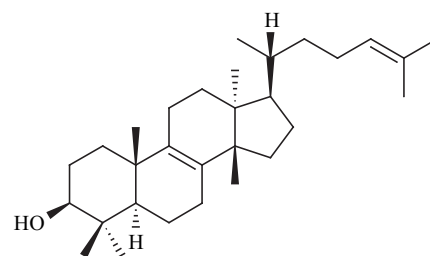
Pachymic acid (**43**), R=Ac, Δ^8
 Dehydrotumulosic acid (**44**), R=H, $\Delta^{7,9(11)}$
 3 β -*p*-Hydroxybenzoyldehydrotumulosic acid (**45**),
 R=*p*-HOC₆H₄CO, $\Delta^{7,9(11)}$



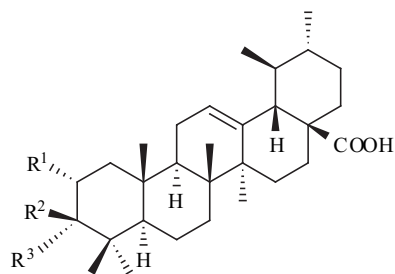
Oleanolic acid (**46**), R= α -H, β -OH
 Oleanonic acid (**47**), R=O



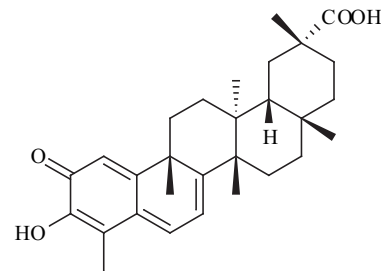
Lupeol (**48**), R¹=H, R²=Me
 Lupeol linoleate (**49**), R¹=linoleyl, R²=Me
 Betulinic acid (**51**), R¹=H, R²=COOH



Euphol (**50**)



Ursolic acid (**52**), R¹=R³=H, R²=OH
 2 α ,3 α -Dihydroxyurs-12-en-28-oic acid (**53**), R¹=R³=OH, R²=H
 2 α -Hydroxyursolic acid (**54**), R¹=R²=OH, R³=H



Pristimerin (**55**)

oleanolic acid which was inactive. Both inhibited the neutrophil infiltration, measured as myeloperoxidase activity, by 84 % and 67 %, respectively. The paw oedema induced by bradykinin was significantly reduced (61 %) by oleanolic acid (**46**) and both compounds also reduced the paw oedema induced by PLA₂ at the dose of 30 mg/kg. Oleanonic acid (**47**) also showed activity on DPP-induced ear oedema, reducing the swelling by 40 %. None of these compounds showed activity on the contact hypersensitivity model of oedema by dinitrofluorobenzene, which suggests that there was no interference with the expression of cell-mediated immunity. When tested on cellular systems, the synthesis of LTB₄ was considerably reduced by oleanonic acid in a dose-dependent manner (IC₅₀: 17 μM).

Among the plant terpenoids with a lupane skeleton [60-61], the *in vivo* anti-inflammatory and anti-arthritis activities of lupeol (**48**) and lupeol linoleate (**49**) have been evaluated in rats. Both compounds (50 mg/kg) showed a reduction in paw swelling by 39 and 58%, respectively in adjuvant arthritis. Moreover, Fernandez *et al.* [62] have evaluated their effects on murine models of acute inflammation. Lupeol (**48**) (0.5 and 1 mg/ear) administered topically suppressed TPA-induced oedema, being less effective on ear oedema induced by AA. Its topical activity was associated with reduction in cell infiltration into inflamed tissues, as demonstrated by quantitation of the neutrophil specific marker myeloperoxidase (MPO) (inhibition percentages of 71 and 78.5 % at 0.5 and 1 mg/ear, respectively).

Huget *et al.* [63] have evaluated the effects of 11 triterpenoids against the mouse ear oedema induced by different protein kinase C activators and neurogenic inflammation elicitors (resiniferatoxin and xylene) in order to establish the mode of their anti-inflammatory activity. The effects on bradykinin-induced paw oedema and on the rat skin inflammation caused by hydrogen peroxide administration were also examined. The oedema induced by mezerein and DPT was reduced to different extents by the triterpenoids assayed (0.5 mg/ear). Lupane and oleanane derivatives were the most effective compounds against DPT-induced oedema. Oleananes and lupanes possessing a carboxyl group were active against byrostatin-1-induced oedema, while the oleanane and lupane alcoholic derivatives were active against the plantar oedema induced by bradykinin and on intradermal inflammation induced by hydrogen peroxide. Most of the triterpenoids were ineffective against the neurogenic inflammation caused by resiniferatoxin and xylene. The authors concluded that the anti-inflammatory activity of triterpenoids might depend on inhibition of protein kinase C, without any involvement of neurogenic inflammatory mechanisms.

More than a dozen of triterpene alcohols and a sterol glucoside isolated from the dichloromethane extract of *Euphorbia kansui* (Euphorbiaceae) were examined for their inhibitory effects on TPA-induced inflammation in mice. All the isolates markedly inhibited oedema formation with a ID₅₀: 0.2-1.0 mg/ear. Euphol (**50**), the most predominant triterpene alcohol constituent exhibited the strongest inhibitory effect (0.2 mg/ear) [64].

Ryu *et al.* [65] reported the *in vitro* anti-allergic and anti-inflammatory activities of four triterpenes: betulinic acid

(**51**), ursolic acid (**52**), 2α, 3α, dihydroxy-urs-12-en-28-oic acid (**53**) and 2α-hydroxyursolic acid (**54**) obtained from the methanolic extract of *Prunella vulgaris* (Lamiaceae). Compound **53** exhibited anti-allergic activity, as assessed by the significant inhibitory effect on the release of β-hexosaminidase from mast cells (IC₅₀: 57 μM). When tested as inhibitors of NO synthesis from LPS-stimulated RAW 264.7 cells, all tested compounds inhibited NO production, showing ursolic acid (**52**) the strongest *in vitro* activity (IC₅₀: 17 μM).

Dirsch *et al.* [66] have described the effects of pristimerin (**55**) on NO production using the same cellular system. This friedelan triterpenoid showed dose-dependently reduced nitrite production (IC₅₀: 0.2-0.3 μM), an effect which correlates with a reduced NOS-2 activity and inhibition of NF-κB activity.

When tested *in vitro*, the pentacyclic triterpene lupeol (**48**) significantly reduced PGE₂ production from A23187-stimulated macrophages, but failed to affect LTC₄ release. It was a weak inhibitor of NO production, but inhibited cytokine production (TNF-α and IL-1β) in LPS-stimulated macrophages. Thus, lupeol (**48**) was able to prevent the production of some inflammatory mediators, which likely contributed to its *in vivo* anti-inflammatory effects [62].

In order to develop new potential molecules, more than 80 novel triterpenoids, all derivatives of oleanolic and ursolic acids were evaluated as potential anti-inflammatory agents [67-70]. They have been tested for their ability to suppress *the novo* expression of two enzymes: NOS-2 and COX-2 in LPS-stimulated RAW 264.7 macrophages. Several molecules were active in these assays, being significantly more active than oleanolic (**46**) and ursolic (**52**) acids. The observed suppression of transcription factor NF-κB activation may partially account for these effects.

CONCLUSION

A vast number of terpenoids have been evaluated as potential anti-inflammatory molecules not only in *in vivo* animal models, but also in well defined *ex vivo* cultures of cells compromised in the inflammatory response, such as monocyte/macrophages, neutrophils, mastocytes and leukocytes. In addition to this, some clues coming from the use of plant extracts rich in these terpenoids and administered in traditional medicine, point to the existence of likely candidates to act as potent anti-inflammatory drugs. In this sense, the plant kingdom has been a potential source of these compounds. However, it has been difficult to define precise molecular motifs, broadly distributed among these terpenes and involved in their anti-inflammatory activity. Some of the terpenoids act as plant hormones regulating different physiological functions (for example the gibberellins), but other are secondary metabolites involved in host defence and in the protection of the plant/animal from potential pathogens. Recent advances in the field of the regulation of innate immunity from insects to mammals have demonstrated the existence of a previously unexpected conservation in the pathways (receptors, kinases and effector molecules) that are involved in this process. From this point

of view, it might be possible to understand the diversity in terpenoids as a wide cross-interaction between plants and potential feeders. Indeed, although immunity and inflammation seem to follow a conserved scheme of regulation among species, the redundancy in the pathways that occurs as the complexity of the organisms increases can explain the loss of efficiency of these defence molecules on higher vertebrates. According to this suggestion, when the molecular targets of anti-inflammatory terpenoids has been analysed in detail, they involved from early steps in the signalling (for example the activation of NIK, a MAPKK kinase) to more distal steps such as the activation of NF- κ B. Finally, the improvement in the knowledge of the precise mechanism that govern the inflammatory reaction may help to unravel the design of modifications that increase the therapeutic efficiency of *a la carte* designed new terpenoids. Once the targets are identified, the use of combinatorial computational methods, as recently developed for the design of inhibitors, can be used successfully in this field [71]. It is to be remembered that some molecules are very active in the low micromolar range.

LIST OF ABBREVIATIONS

AA	=	Arachidonic acid
COX	=	Cyclooxygenase
EPP	=	Ethylphenylpropiolate
DPP	=	12-deoxyphorbol-13-phenylacetate
DPT	=	12-deoxyphorbol-13-decanoate
IFN- γ	=	Interferon-gamma
IL-1	=	Interleukine-1
LOX	=	Lipoxygenase
LPS	=	Bacterial lipopolysaccharide
LTs	=	Leukotrienes
MPO	=	Myeloperoxidase
NF- κ B	=	Nuclear factor-kappa B
NMR	=	Nuclear Magnetic Resonance
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
PGs	=	Prostaglandins
PKC	=	Protein kinase C
PLA ₂	=	Phospholipase A ₂
TPA	=	12- <i>O</i> -tetradecanoylphorbol-13-acetate
TNF- α	=	Tumour necrosis factor
TXs	=	Thromboxanes

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