CHAPTER 11

PLANT SECONDARY METABOLITES. TARGETS AND MECHANISMS OF ALLELOPATHY

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INTRODUCTION

Allelopathy is commonly defined as any effect: direct or indirect, stimulatory or inhibitory, mediated by a chemical compound released into the environment by a given plant or microorganism (Rice, 1984). These chemicals, termed secondary metabolites, allelochemicals**,** natural products or phytogrowth-inhibitors, are a major factor in regulating the structure of plant communities (Smith and Martin, 1994). Allelochemicals can be released into the environment through a variety of mechanisms: volatilization from leaves, exudation from roots, and leaching from fallen leaves and plant litter (Putnam, 1983).

The chemical interaction between plants was known for thousands of years, before the term allelopathy was coined by Molish (1937). Thus, Pliny may have been the first to record the allelopathic effects of the walnut tree (*Juglans nigra* and *J. regia*). The walnut tree is perhaps the best known allelopathic plant, causing many crops and other plants in its vicinity to wither and die. The leaves, roots, and fruits of the plant produce a hydroquinone (1), which is oxidized in the environment to juglone (2), the compound responsible for the toxic effects on other plants (Kocacaliskan and Terzi, 2001).

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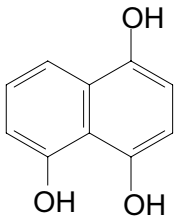

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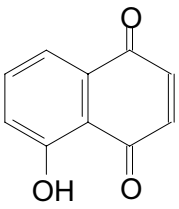

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Secondary metabolites

According to Whittaker (1970) and Whittaker and Finney (1971), most allelochemicals are secondary metabolites, not present in all living organisms but appearing sporadically. The production of allelochemicals is promoted by biotic and abiotic stress. Allelochemicals are referred to as secondary metabolites due to the fact that they are obtained through branching of the main metabolic pathways of carbohydrates, fats and aminoacids. Whittaker and Finney (1971) listed the chemical character of allelopathic molecules as belonging to several major groups: these include phenolic acids, flavonoids, and other aromatic compounds, terpenoid substances, steroids, alkaloids, and organic cyanides. Rice (1984) devised a classification system containing 14 chemical categories plus a miscellaneous group. Most allelochemicals derive either from acetate, or from aminoacids participating in the shikimic acid pathway. The categories proposed by Rice (1984) are (1) cinnamic acid derivatives, (2) coumarins, (3) simple phenol or benzoic acid derivatives, gallic acid and protocatechic acid, (4) flavonoids, (5) condensed and hydrolyzable tannins, (6) terpenoids and steroids, (7) water-soluble organic acids, straight chain fatty acids, (10) naphthoquinones, anthraquinones and complex quinones, (11) aminoacids and polypeptides, (12) alkaloids and cyanohydrins, (13) sulfide mustard oil glycosides and (14) purines and nucleosids. From the structural diversity of the allelochemicals, it is obvious that allelopathy must involve more than one action mechanism, different synergism patterns and diverse targets of interaction. Thus a large diversity of molecules may be allelochemicals, but not all the compounds belonging to each of the categories in the Rice classification must be allelochemicals. Furthermore, in addition or instead of acting as allelopathic agents, many of these compounds may protect plants against herbivores or against microbial infection, or function as attractants for pollinators and seed-dispersing animals.

It is a common practice to determine the allelochemical threshold concentration in susceptible plants. Allelopathy is a concentration dependent phenomenon, and allelochemicals are introduced into the environment together with a vast number of

other secondary metabolites. Thus, it is likely that synergistic effects enhance the observed activities (Putnam et al. 1986). The kinetics activity of allelochemicals is also studied.

Current allelopathy research is interdisciplinary and to make significant progress, it requires the contribution of agronomists, biologists, biochemists, ecologists, organic chemists, physiologists, soil scientists, and theoretical chemists.

Even when a given allelochemical may be tentatively identified, it is exceedingly difficult to probe its role in plant-plant interactions. Even more difficult to analyze is the primary site and the mechanism of action and to distinguish these from secondary site(s) or action mechanism(s). Measurement of the amount of an allelochemical released and absorbed is likewise complicated by interactions of the molecule with soils of different types, and by degradation or modification of the compound by microorganisms. Often, only minute quantities of the active compound can be isolated from the natural source. The participation of volatile natural products in plant-plant interactions may be easier to be demonstrated and so is the analysis of the structure.

Biological assays

The most widely used biological assays for allelochemicals are seed germination and seedling growth studies. In the simplest form of these assays, seeds of the selected plant species are placed on filter papers or on agar in a Petri dish or in a small tissue culture and treated with a solution of the suspected allelochemical at various concentrations. Germination rate and seedling growth (root and shoot lengths) is then monitored versus control samples. The most important consideration in choosing or developing a bioassay for an allelopathic study is selection of the target species from both mono and dicotyledons in order to determine the potential selectivity of the allelochemicals. The most used model for weeds is lettuce (*Lactuca sativa* L.). It has been used extensively as a test organism because of its fast germination and high sensitivity (Rasmussen and Einhelling, 1979). *Lemna* sp. (duckweed) is often used to examine plant-plant interactions in aquatic environments (Elakovich, 1999), while barnyardgrass, gooseweed, and ducksalad are more relevant for study of allelopathy in rice (Dilday et al. 2000). The initial screening can be done using dose-response curves for concentration of the allelochemicals ranging from 10^{-4} to 10^{-9} M.

Target(s) and mechanisms of action of allelochemicals

It is naive to expect that the physiological action of the numerous allelochemicals will be the same. Nonetheless, most investigations have used the common denominator

that deleterious effects in allelopathy will be expressed through a reduction or delaying of germination and stunting of growth of a susceptible plant. Apparently, allelochemicals may alter a variety of physiological processes and it is difficult to separate primary from secondary relationships when interpreting the effect of these phytotoxins on plant growth or seed germination. A further complication is that the various allelochemicals may inhibit through different mechanisms. Separating primary from secondary effects can be challenging when nothing is known about the mode of action of the compound.

The morphology of grown seedlings in the presence of allelochemicals may also yield important information. In particular, abnormal root formation such as clubbing may indicate a mode of action of compounds that are inhibitors of microtubule assembly. Stimulation of lateral root growth to the detriment of the primary root also suggests disruption of hormonal balance. Investigation of the putative effect on root cell division should be conducted by measurement effects of the compound on the mitotix index with plant roots. Subjective biological observations include root and shoot morphology and visual rating of the phytotoxic effect. Objective quantitative measurements include measurement of the length and weight of the plant parts. Leaf area measurements can provide information related to the inhibition of plant development.

Therefore, the study of physiological and biochemical roles of secondary metabolite potential targets, include: *modification of membrane structure and all type of transport receptor vs. signalling, altered features of cell morphology, interference with the cell cycle (replication, protein synthesis, mitosis), modification of phytohormone activity, perturbation of energy metabolism (respiration and photosynthesis), modification of water balance and stomata function, inhibition of pigment synthesis and/or degradation, and blockage of function for a number of enzymes, mainly regulatory enzymes.*

MEMBRANE STRUCTURE AND TRANSPORT AS TARGET OF ALLELOCHEMICALS

Cell membranes consist of a bilayer of polar lipid molecules and associated proteins. In most cell membranes, lipids and proteins (glycoproteins) make roughly equal contributions to the membrane's mass. The lipids belong to several classes, including phospholipids, galactosylglycerides, glucocerebrosides, and sterols. These molecules share an important physicochemical property: they are amphipathic, containing both hydrophilic (water-loving) and hydrophobic (apolar-loving or water-hating) domains. When brought into contact with water, these molecules spontaneously self-assemble into higher-order structures. The hydrophilic head groups maximize their interactions with water molecules, whereas the hydrophobic tails

interact with each other, minimizing their exposure to the aqueous phase. For most membrane lipids, the bilayer configuration is the minimum-energy self-assembly structure. In this configuration, the polar groups form the interface to the bulk water, and the hydrophobic groups become sequestered in the interior. Studies of the movement of phospholipids in bilayers demonstrate that each phospholipid can diffuse laterally, rotate, flex its hydrophobic tail, bob up and down and flip-flop. Membrane lipids exist in two different physical states: as a gel and as a fluid. The change in state is known as phase transition. Gelling brings most membrane activities to a standstill and increases permeability.

Many plants survive temperature fluctuations of up to 30 ºC on a daily basis. How do organisms adapt the fluidity of their membranes to suit their mutable growth environments? Membrane sterols serve as membrane fluidity "buffers", increasing the fluidity at lower temperatures by disrupting the geling of phospholipids, and decreasing fluidity at high temperatures by interfering with the flexing motions of the fatty acid tails. Like all cellular molecules, membrane lipids have a finite life span and have to be turned over on a regular basis. This turnover also enables plant cells to adjust the lipid composition of their membranes in response to seasonal changes in ambient temperature. Allelochemicals with detergents properties may solubilize phospholipid(s) and perturb the membrane function mimicking sterol-behavior and thus causing growth inhibition. Allelochemicals can also alter the fluidity of the membrane and thus modify the transport processes that need fluidity. There can be a direct inhibition of the interaction between carriers or transporters and the secondary metabolites resulting in transport inhibition.

Membrane proteins associate with lipid bilayers. The fluid-mosaic membrane model included two basic types of membrane proteins: peripheral and integral protein. Most recent research has led to the discovery of four additional classes of membrane proteins: fatty acid-linked, prenyl group-linked, phosphatidylinositol – anchored, and cholesterol-linked, all of which are attached to a bilayer by lipid tails. Peripheral proteins are water-soluble and can be removed by washing membranes in water or in salt or acid solutions that do not disrupt the lipid bilayer. Peripheral proteins bind either to integral proteins or to lipids through salt bridges, electrostatic interactions, hydrogen bonds or some combination of these. Some peripheral proteins also provide links between membranes and cytoeskeletal systems. In contrast, integral proteins are non soluble in water. Because at least one domain lies embedded in the hydrophobic interior of the bilayers, an integral protein can be removed and solubilized only with the help of detergents or organic solvents, which degrade the bilayer.

Both the fatty acid-linked and the prenyl group-linked proteins bind reversibly to the cytoplasmic surfaces of membranes to help regulate membrane activities. Cycling between the membrane-bound and free states is mediated in most cases by phosphorylation/dephosphorylation or by GTP/GDP binding cycles.

Membrane fluidity

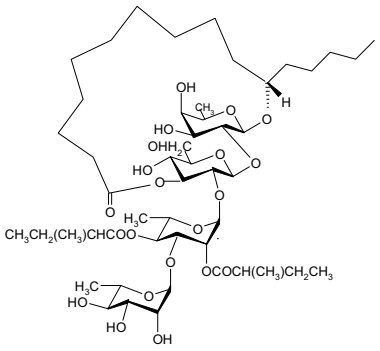
Involves the movement of not only lipid molecules but also integral proteins that span the bilayer and of different types of surface-associated membrane proteins. Collisional interactions are essential for the transfer of substrates between many membrane-bound enzymes and for electrons passage from between the electron transfer chain components of chloroplasts and mitochondria. Such movements are also critical for the assembly of multiprotein membrane complexes. In addition, many signalling pathways depend on transient interactions among defined sets of integral membrane proteins and peripheral or lipid-anchored proteins. These multiprotein assembly can be altered with allelochemicals and inhibits the normal membrane processes.

Tethering structures regulate and restrict the movement of membrane proteins, often limiting their distribution to defined membrane domains. This tethering can involve connections to the cytoskeleton and the cell wall, bridges between related integral proteins, or junction-type interactions between proteins in adjacent membranes.

A particularly striking example of the junction type of interaction occurs in the grana stacks of chloroplast membranes. Grana stack formation has been shown to affect the lateral distribution of all major protein complexes in thylakoid membranes and to regulate the function of the photosynthetic reaction centers and other components of the photosynthetic electron transport chain of PSII and PSI. Modification of the lateral distribution in the PSII complexes can affect the rate of electron transport in photosynthesis.

In order to eliminate weeds prior to planting the next sugar cane crop, *Ipomoea tricolor* is used as cover crop (another way to find allelopathic plants) in the State of Morelos, Mexico, because it possess allelopathic properties. Bioassay-guided analysis of *I. tricolor* extracts yielded tricolorin A (3) with a potent phytogrowth inhibitory activity. In addition, this macrolactone oligosaccharide has antibiotic and antitumor activities (Pereda-Miranda et al. 1993). Some minor components of the mixture, tricolorins B-E were identified by FAB-Mass spectrometry and high resolution of detailed NMR analysis (Bah and Pereda-Miranda, 1996). Furthermore, studies on the inhibition of plasma membrane H^+- ATPase by the glycoside mixture indicated that **3** was the inhibitor compound (Calera et al. 1995). Petri dish bioassays combined with greenhouse experiments suggested that the weed suppressive activity of *I. tricolor* may involve both leaching of allelochemicals from the living plant by rain and release of allelochemicals from decaying plant matter (Anaya et al. 1995). Further studies indicate that Tricolorin A (3) behaves as uncoupler (U_{50} = 330 nM) in spinach chloroplasts, because it inhibited H^+-uptake and ATP synthesis, and stimulates basal and phosphorylating electron flows. At higher concentrations of tricolorin A,

inhibition of photosystem II electron transport at the level of Q_B (I_{50} = 5 μM) occurred. Chlorophyll *a* fluorescence analysis corroborated this finding and that the macrolactone is a structural requirement for this activity (Achnine et al. 1999). Therefore, the mechanism of action of tricolorin A (3) as allelopathic compound is by uncoupling photophosphorylation and interacting at plasma membrane H^+-ATPase. The novel macrolactone structure of the tricolorins has attracted the attention of the synthetic chemists (Larson and Heathcock, 1997; Lu et al. 1997).



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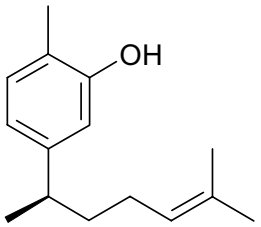
Iostephane heterophylla (Cav) Hemsl. (Asteraceae) is widely distributed throughout Mexico, from the states of Chihuahua and Sinaloa in the north to Oaxaca in the south. Xanthorrhizol (4), a sesquiterpene of the bisabolene type, has been isolated from the roots of this plant (Aguilar et al. 1993; Mata et al. 2001) in addition to other sesquiterpenes, diterpenes, 8-hydroxy-6-acetyl-2, 2-dimethylchromene and scopoletin. Xanthorrhizol (4) possess insecticidal activity against neonate larvae of *Spodoptera littoralis* (Pandji et al. 1993). González-Bernardo et al. 2003, found the phytotoxic effect of xanthorrhizol on photosynthesis by inhibiting ATP synthesis and electron flow (I_{50} = 31 μM), the site of inhibition is located at the span of P_{680} to Q_A and PQ as was corroborated by fluorescence decay data. Acetylating of xanthorrhizol indicates that the OH group is essential for interaction with the electron transport chain in the thylakoid, since 1-O-acetyl-12, 13-dihydro-xanthorrhizol (5) is almost inactive. Furthermore, the reduction of the $C_{12\text{-}13}$ double bond may facilitate the interaction with the target redox electron transport chain or the penetration to the site of interaction, making the molecule more soluble in the lipid phase explaining why this is the most active derivative. The reduced xanthorrhizol also inhibits the H^+-ATPase. A bioguided fractionation of *Iostephane heterophylla* also led to the isolation of trachyloban-19-oic acid (6) as the compound that inhibits PSII at the level of Q_B (I_{50} = 25 μM) and the

perturbation of LHC II. Chlorophyll fluorescence studies confirm the behavior of this diterpene (Hernandez-Terrones et al. 2003a). In order to understand which part of the structure is important for inhibition, methyl trachyloban-19-oate ester (7) was prepared from trachyloban-19-oic acid with ethereal diazomethane (Hernandez-Terrones et al. 2003b), this derivative presented a new site of interaction, acting as energy transfer inhibitor, blocking CF_0 channel of the chloroplast ($I_{50} = 10$ μM), because increasing concentrations of methyl trachyloban-19-oate ester restore the light dependent pH rise to a suspension of EDTA-washed chloroplasts. Methyl trachyloban-19-oate ester (7) is a noncompetitive inhibitor to well known energy transfer inhibitors DCCD and triphenyltin, therefore it has a unique site of interaction at CF_0 channel.



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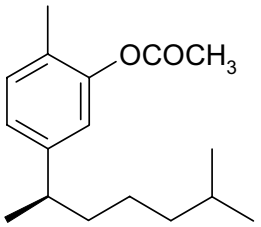

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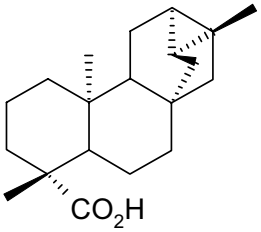

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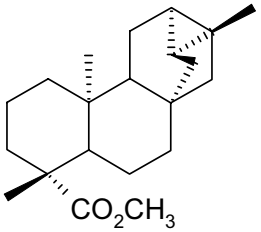
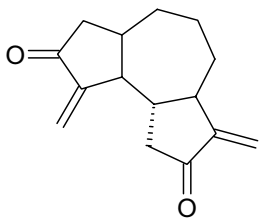


(7)

Parthenium hysterophorus is an aggressive, neotropical composite weed that has spread very fast in the world. It exerts negative effects on agriculture, animal husbandry, ecology and environment (Evans, 1997). Its allelopathic properties have been demonstrated (de la Fuente et al. 2000). These are attributed to the presence of parthenin (8), a sesquiterpene lactone of pseudoguayanolide nature found in various parts of the weed (de la Fuente et al. 2000). The compound is sequestered in trichomes that cover the whole plant (Picman and Picman, 1984). Parthenin is known to be phytotoxic against many plants (Batish et al. 2002). Germination of *Avena fatua* and

Bidens pilosa was reduced with parthenin (I_{50} = 625 and 700 μM, respectively). Further, parthenin also inhibited the growth of both weeds in terms of root and shoot length and seedling dry weight. Inhibition of root growth was greater than that of shoot growth. In addition, parthenin caused reduction of chlorophyll content and water loss in the weedy species. The site of interaction and mechanism of action is unknown.

The following secondary metabolites isolated from Mexican plants: encecalin (9), and demethylencecalin (10) (Castañeda et al. 1996), cacalol (11), cacalol methyl ether (12), cacalol acetate (13), 2-acetylcacalol acetate (14) (Lotina-Hennsen et al. 1991), ivalin (19), zaluzanin C (20) isoalloalantolactone (21) (Lotina-Hennsen et al. 1992), odoratol (22), α-photogedunin acetate (23), β-photogedunin acetate (24) (Céspedes et al. 1998), and phenylpropanoid 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (25), methyl-ester of 3,4-dihydroxy-trans-cinnamic acid (26) has been found to act as Hill reaction inhibitors in chloroplasts because they inhibit electron flow (basal, phosphorylation and uncoupled), proton uptake and ATP synthesis. Polarographic analysis of the photosynthetic partial redox reactions indicate that, uncoupled PS I electron flow and uncoupled PS II electron transport from DPC to DCPIP of Tris washed thylakoids were insensitive to cacalol (11), ivalin (19), zaluzanin (20), doratol (22) (Achnine et al. 1998) and methyl-ester of 3,4-dihydroxy-trans-cinnamic acid (26) but they inhibited uncoupled PS II electron flow as measured from water to SiMo or DAD, and from H_2O to DCPIP at the same extent. These results indicate that the target site for these compounds is located at the water splitting enzyme.



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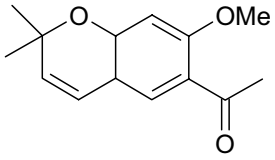
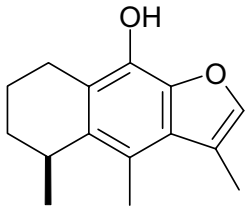


(9): R = Me
(10): R = OH



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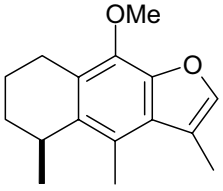

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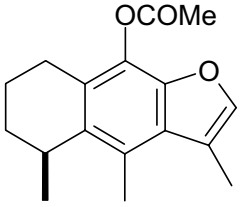

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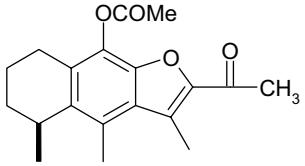

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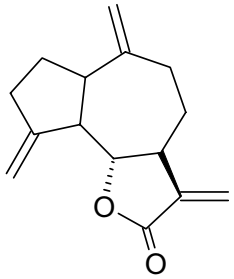

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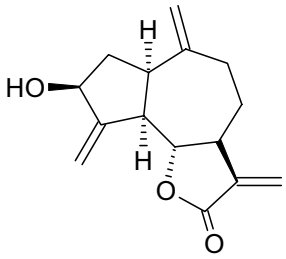

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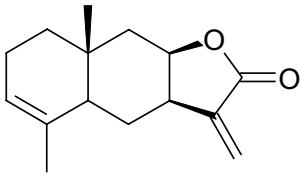

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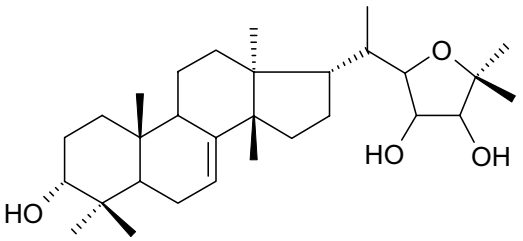

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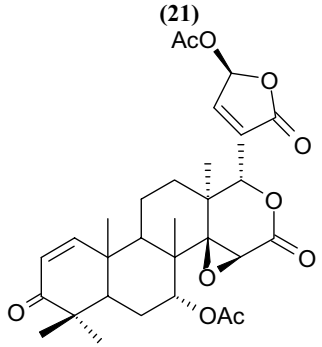

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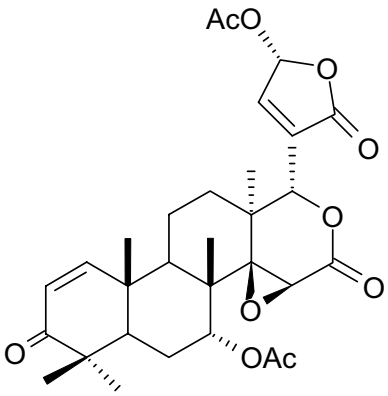

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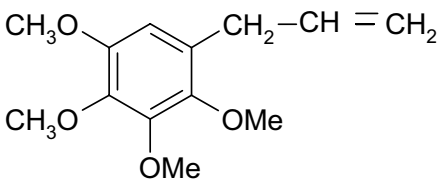

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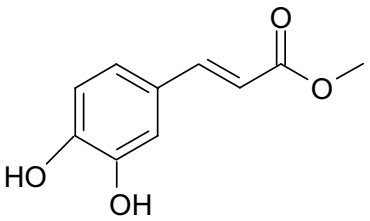


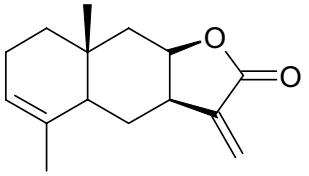
(26)

The most important findings for odoratol (22) are: (1) the natural product was found to be an inhibitor (150 μM) of oxygen evolution. (2) The diol moiety at positions 23 and 24 is an important structural requirement for the inhibitory activity displayed by (22). (3) Chlorophyll a fluorescence measurements revealed a pattern similar to that resulting from hydroxylamine and Tris treatments, both well known inhibitors of the water-splitting enzyme, the presence of (22) induces the formation of a faster transient "K" between 0.8 and 1.0 ms and the formation of a quencher after 0.8 ms. These data corroborate the results obtained by polarography measurements.

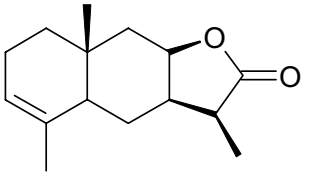
To get further evidence about the cacalol inhibition site, the redox behaviour of cacalol and its semisynthetic derivatives were studied using cyclic voltammetry. The voltammograms in a protic solvent were indicative of an electrochemically irreversible oxidation of the phenolic system. The electrooxidation of compounds 11-14 in an aprotic solvent was irreversible with similar oxidation patterns and more complex than in the protic solvent but presented similar oxidation patterns. Compounds containing an unsubstituted phenolic system presented a redox potential of Epa = + 0.824 V (V vs NHE), which resembles the potential for the water-splitting enzyme of thylakoid membrane. This result explains why cacalol inhibited the water splitting enzyme complex.

On the other hand, the site of interaction of compounds which act as Hill Reaction inhibitors, such as encecalin 9, and demethylencecalin 10 (Castañedaet al. 1998), cacalol 11**,** cacalol methyl ether 12, cacalol acetate 13, 2-acetylcacalol acetate 14, isoalloalantolactone 21, and 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 25, is located in the span from P_{680} to Q_A (Jiménezet al. 1998), of the non-cyclic electron transport chain of thylakoids. Furthermore, the uncoupled electron transport from water to DCPIP (or DAD), from water to SiMo and, from DPC to DCPIP is inhibited by these compounds to the same extent. However, uncoupled PSI electron transport from $DCPIP_{red}$ to MV is unaffected by these metabolites. The level of activity displayed by compound 25 (IC_{50} is 2.7 μM) is remarkable as compared to other Hill reaction inhibitors, including commercial herbicides. Therefore, this simple phenylpropanoid represents an important lead for development of new "green" herbicides agents.

The comparison of the inhibitory effect on ATP synthesis induced by compounds 27, 28, 29, and 30 clearly indicates that the $\Delta^{3,4}$ double 0 of isoalloalantolactone is an essential structural requirement for its inhibitory effect on photophosphorylation. However, the α-methyl-γ-lactone moiety was important but not essential for ATP synthesis inhibition.



(27)



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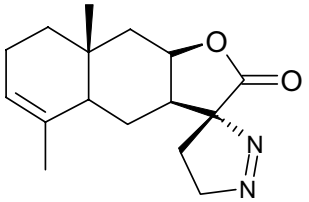

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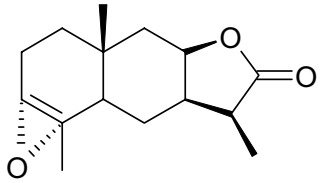


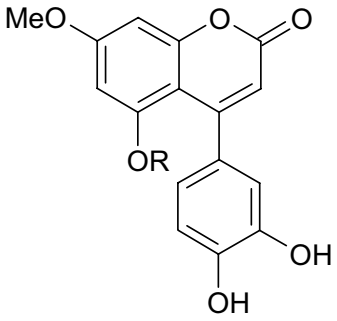
(30)

α-Photogedunin acetate 23, inhibits PSII electron transport from water to DCBQ without affecting PSI electron transport from $DCPIP_{red}$ to MV and PSII electron flow from water to SiMo. Thus, the target of α-photogedunin acetate (23) is localized at Q_B level. However, β-photogedunin acetate (24) inhibits partially, and with a similar potency of inhibition the PSI electron flow from $DCPIP_{red}$ to MV and from PMS_{red} to MV. The electron flow inhibition from $TMQH_2$ to MV is similar to the inhibition of electron flow from DCPIP to MV; thus, it indicates that 24 interacts at b_6f complex and in the span of P_{700} to F_x. The overall results indicate that the stereochemistry at C-23 determines the target site for interaction at the thylakoid electron transport chain. Furthermore, it is important to point out that photogedunin 23 and 24 only inhibited the redox enzymes on the thylakoid membranes when the assay media were preilluminated with actinic light during 3 minutes.

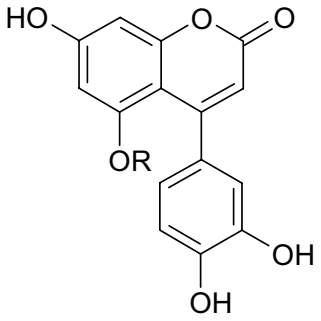
Uncouplers

The natural 4-phenylcoumarins 31-38 (Calera et al. 1996) and some semisynthetic derivatives as well as the sesquiterpene lactones dehidrocostuslactone 39 and

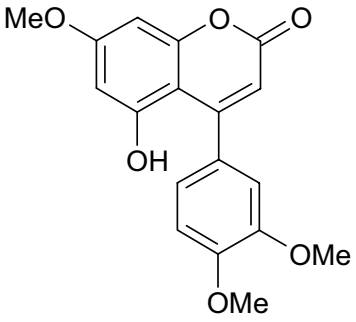
costunolide 40 acted as uncouplers in spinach chloroplasts. The glycoside 5-O-β-D-glucopyranosyl-7-methoxy-3',4'–dihydroxy-4-phenyl-coumarin 35, 7-methoxy-5,3',4'-trihydroxy-4-phenylcoumarin 33 inhibited ATP synthesis and proton uptake. On the other hand, these compounds enhanced basal and phosphorylating electron transport. The light-activated Mg^{2+} -ATPase was slightly stimulated by coumarins 32 and 33. In addition, at alkaline pH compound 32 stimulated the basal electron flow from water to methylviologen, but at the pH range from 6.0 to 7.5 the coumarin did not have effect. Compound 32, which possesses four free phenolic hydroxyl groups, was the most active uncoupler agent. Methylation of 36, and 37 at the phenolic groups at C-3', C-4', and C-5 resulted in a reduction or loss of the uncoupling activity. Therefore, the phenolate anions may be the active form responsible for the uncoupling behaviour of 4-phenylcoumarins.



(31): R = -β-D-galactopyranosyl
(33): R = H
(35): R = - β-D-glucopyranosyl



(32): R = H
(34): R = - β-D-glucopyranosyl



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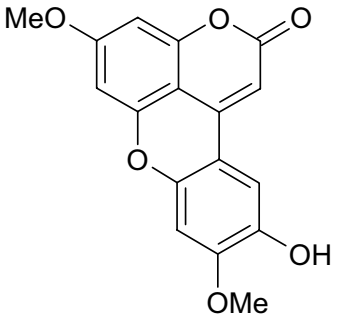

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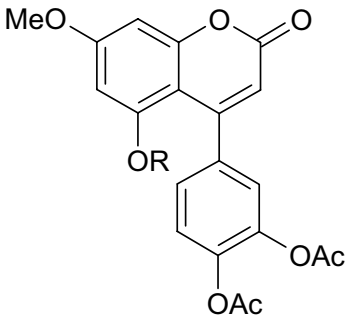
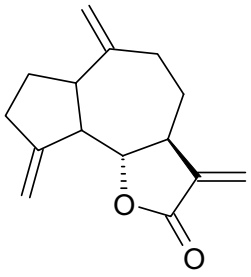


(38): R = tetra-acetyl-β-D-galactopyranosyl



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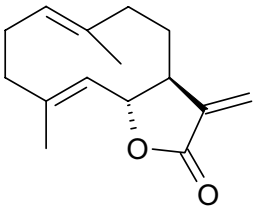

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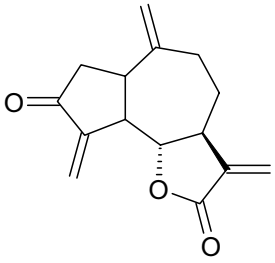


(41)

Plasma membrane

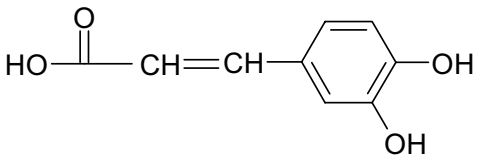
It forms the outermost boundary of the living cell and functions as active interface between the cell and its environment. In this capacity, it controls the transport of molecules into and out of the cell, transmits signals from the environment to the cell interior, participates in the synthesis and assembly of the cell wall molecules, and provides physical links between elements of the cytoskeleton and the extracellular matrix. In conjunction with specialized domains of the ER, the plasma membrane produces plasmodesmata; membrane tubes that cross cell walls and provide direct channels of communication between adjacent cells. As result of these plasmodesmal connections, almost all the living plant cells of an individual plant share a physical continuous plasma membrane.

In plant plasma membranes, the ratio of lipid classes varies remarkably among the different organs in a given plant and among identical organs in different plants. Barley root cell plasma membranes contain more than twice as many free sterol molecules as phospholipids. In barley leaf plasma membranes, the phospholipids-to-free sterol ratio is 1:3:1, whereas in spinach it is 9:1.

Most plasma membrane proteins involved in transmembrane activities such as transport and signalling, the anchoring of cytoskeletal elements to cell wall molecules, and the assembly of cellulose fibrils from cytosolic substrates are of the integral type. However, those proteins often form larger complexes with peripheral proteins. The extracellular domains of many integral proteins are glycosylated, bearing N-and O-linked oligosaccharides.

The plasma membrane H^+-ATPase (P-type H^+-ATPase) is the principal primary active transport system of plant cells. For example, two plant cell systems regulate the uptake of K^+, which is required for growth and osmoregulation. One system corresponds to a low-affinity K^+-uptake channel and a second to a high-affinity, H^+-gradient-dependent K^+-uptake carrier. Dehydrozaluzanin C (41) is a sesquiterpenol, with a guaiane skeleton that has been isolated from the roots of many different compositae families. It inhibits root growth and germination of plants. The I_{50} value for lettuce root growth is 500 μM (Galindo et al. 1999). (41) **C**auses also rapid plasma membrane leakage in cucumber cotyledon discs. It is more active at 50 μM. Notice that the concentration needed for this activity is 10X less (41) symptoms include plasmolysis and disruption of membrane integrity, is not light dependent. Its effects on roots were reversible through the addition of various aminoacids, with histidine and glycine providing ca 40% reversion. However, a strong reversal effect was obtained with reduced glutathione, as a result of gross-reactivity with (41), it has no-effect on photosyntesis, respiration, mitotic processes and NADH oxidase activity. Thus, (41) exerts its effects on plants through at least two different mechanisms, only one which is related to the disruption of plasma membrane function. On which plasma membrane function acts dehydrozaluzanin C, still needs to be defined. Maybe a competition experiment in plasma membrane leakage with aminoacids vs (41) will give some light. Plant cell plasma membranes also contain proteins known as aquaporins, which form water channels. The leafy spurge (*Euphorbia esula*) displaces many native plant species, it has been demonstrated that small everlasting (*Antennaria microphylla*), a native perennial, is allelopathic to leafy spurge. Isolation of allelochemicals from small everlasting yields hydroquinone, a simple phenolic compound, arbutin, the monoglucoside of hydroquinone and caffeic acid **(42)**, a phenolic acid. Both hydroquinone and **(42)** are inhibitory to leafy spurge seed germination, root elongation and callus culture growth. Results indicate that inhibition of growth in leafy spurge after exposure with **(42)** for 12 days had significantly higher leaf diffusive resistances than control plants. Transpiration was similarly affected with treated plants showing higher transpiration rates compared to controls. Chlorophyll fluorescence was significantly lower in treated plants than controls. The partial closure of the stomates of leafy spurge treated with 250 μM **(42)**, the stable carbon isotope ratio (^{13}C ^{12}C) was higher than controls. These data show that a disruption of plant water relations is the primary mechanism of plant growth inhibition. The water channel aquaporin, is a suggestive target for caffeic acid.

The identification and physiological characterization of several plasma membrane receptors that bind hormones, oligosaccharides, peptides, and toxins are present at low concentrations, and thus are difficult to study. Therefore, many researchers have adapted a genetic approach to identifying and isolating receptor-protein genes.



(42)

Transport is made possible by membrane-spanning proteins within the lipid bilayer. The transport systems can be regarded as conventional enzymes in almost all respect, with the important exception that transport events are vectorial (i.e. defined by a magnitude and a direction in space). Like enzymes, all transport systems exhibit some degree of substrate-specificity and work by lowering the activation energy required for transport. In plants cells, membrane transport underpins a wide range of essential processes like:

Turgor generation. The presence of a cell wall in the vast majority of plant cells enable them to generate turgor (positive pressure). The cell wall provides structural rigidity. Turgor generation is accomplished by accumulating salts. In the mature cells of most plants, K^+ accumulates in the cytoplasm and in the large central vacuole. The cations must be balanced by a corresponding concentration of anions to achieve electroneutrality; in the vacuole, the principal anion is Cl^- or malate.

Plants synthesize organic biomolecules from inorganic nutrients. These must be absorbed from the soil by roots for assimilation into amino acids and other metabolites, i.e. NH_4^+, NO_3^-, $H_2PO_4^-$, SO_4^{2-}, trace elements such as: boron, zinc, copper, and iron, and each probably requires a specific transport system. The metabolism generates waste products that must be removed from the cytosol, i.e. H^+, OH^- produced during assimilation of HCO_3^- and NO_3^- into organic compounds. To expel H^+ from the cytosol, plants have evolved proton pumps at both the vacuolar and plasma membranes. Compartmentalization of metabolites in the cell enhances metabolic efficiency. In the mitochondrial matrix the ADP/ATP and NADH/NAD^+ ratio are greater than in the cytosol, this is possible thanks to specific carrier or transporters and membrane compartmentalization, which provides a substrate/product ratio that favors respiratory activity, the electron flow coupled to

ADP phosphorylation, and O_2 reduction to H_2O. Allelochemicals may interact with one of these processes.

Energy transduction

Membrane transport lies at the heart of the conversion of free energy into biologically useful forms. Light energy stimulates the photosynthetic electron transport chain to pump H^+ into the thylakoid lumen. Similarly oxidation of NADH by mitochondria provides the energy for pumping H^+ from the matrix into the intermembrane space. In each case, the spontaneous exergonic flow of H^+ back across the membrane is used to generate ATP. Understanding the mechanisms by which light and high-energy electrons are harnessed to phosphorylate ADP therefore demands knowledge of membrane transport. The energy production is a primary target for allelochemical.

Natural occurring Annonaceous acetogenins (*ACG*) are present in the seeds of commercially-exploited tropical fruits such as 'guanabana' (*Annona muricata*) and 'cherimoya' (*Annona cherimolia*). *ACG* have also potent activity as insecticides, acaricides, fungicides, antiparasitics, herbicides and inhibitor to tumor cell growth. These compounds belong to a wide group of natural products isolated from several species of the Annonaceae family, which include more than 250 molecules with diverse chemical structures (Zafra-Polo et al.1996a, 1996b). The mode of action of the *ACG,* the main targets are in the mitochondrial NADH: ubiquinone oxidoreductase, also known as respiratory complex I of mitochondria (Zafra-Polo et al. 1996a, 1996b; Esposti et al. 1994).

Complex I transfers electrons from NADH to ubiquinone and links this process to the translocation of protons across membrane generating an electrochemical gradient that drives the synthesis of ATP. Inhibition of complex I opens an interesting perspective for the development of a new generation of antitumor drugs with this particular mode of action.

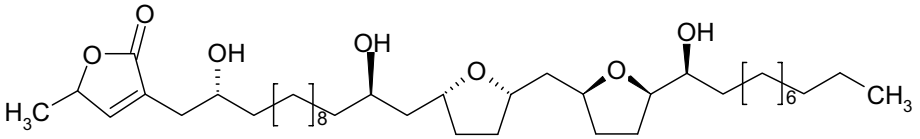
Most studies with *ACG* are done by determination of their cytotoxicity for several human-tumor cell lines (Oberlies et al. 1995, 1997). Nevertheless, growth inhibition of tumor cell cultures depends on many additional factors other than the mode of action of the molecule, such as the intracellular distribution and the diffusion across biological membranes. Studies on the target enzyme are required to better understand the mode of action of these molecules.

Tormo et al. (1999) established the structure-activity relationship (SAR) of Annonaceous acetogenins of the mono-tetrahydrofuranic (mono-THF) type, to perform the inhibitory studies on mitochondrial NADH: ubiquinone oxidoreductase, and NADH oxidase activities using complex I of submitochondrial particles (SMP).

The six mono-THF acetogenins present a common skeleton of 32 C with one α-α'-dihydroxylated THF system and one unsaturated methyl-γ-lactone moiety. They only differ by the number of hydroxyl and carbonyl groups at the aliphatic chain that links the lactone and the THF.

The results show that electron transfer inhibition, when complex I operates in physiological conditions (NADH oxidase activity) is in the same range than the most potent bis-THF *ACG*. However, they were less effective to inhibit the NADH: ubiquinone oxidoreductase activity measured with decylubiquinone as artificial electron acceptor. All mono-THF acetogenins showed values between those of rolliniastatins-I (43) (0.6 nM) and rotenone (5.1nM) against the aerobic oxidation of the NADH. Moreover, annonacinone (44) (Morré et al. 1995) gave an inhibitory potency very close to that of rolliniastatin-I, an *ACG* that belongs to the subclass of bis-THF with *threo/cis/threo/cis/erythro* relative configuration.

All six mono-THF acetogenins act as noncompetitive inhibitors confirming this displacement. Most *ACG* belong to this functional type of inhibitors (Zafra-Polo et al. 1996a, 1996b; Miyoshi et al.1998), but only a few acetogenins show an uncompetitive pattern (Esposti et al. 1994; Estornell et al. 1997). However, all Annonaceous acetogenins have not been studied their role on plant mitochondria, as well as, in chloroplasts, with the exception of Chávez et al. (2001), found that the bis-tetrahydrofuran Annonaceous acetogenins (squamocin (45), bullatacin (46) and motrilin (47)) behave as uncoupler-Hill reaction inhibitors and at higher concentrations these compounds disrupt the interactions between the antenna complexes and interact with PSII.



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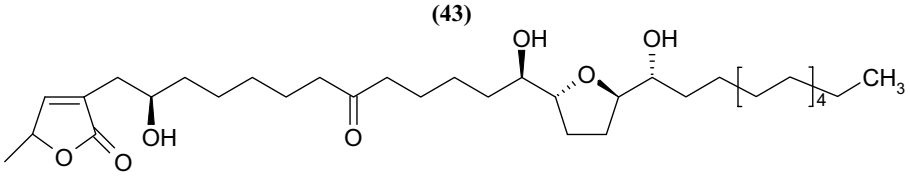

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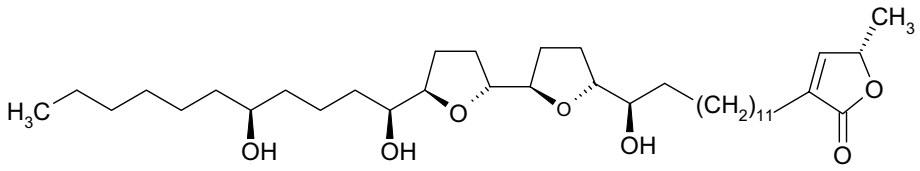

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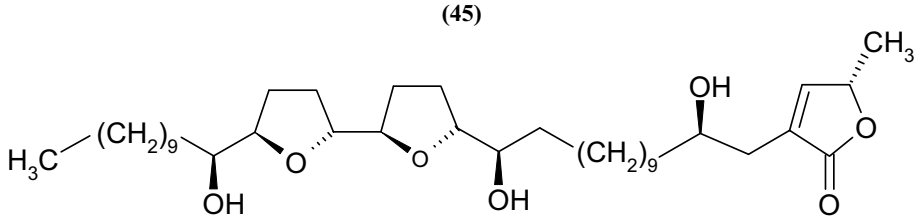

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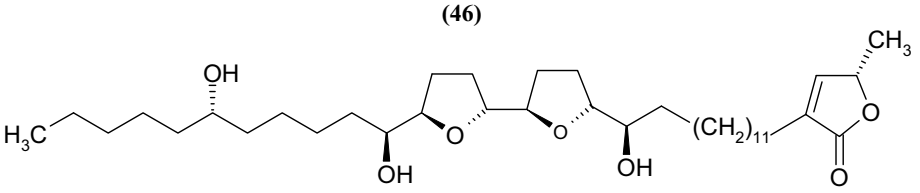


(47)

Signal transduction

Many biotic and abiotic signals for plant growth and development trigger their respective responses by transiently increasing the concentration of cytosolic free Ca^{2+}, carried out by Ca^{2+} translocating ATPases (remove Ca^{2+} from the cytosol by pumping it across the plasma membrane and intracellular membranes) and Ca^{2+}-permeable channels (open in response to particular stimuli and allow the passive entry of Ca^{2+} into the cytosol), thereby propagating the signal.

Organization of transport at plant membranes

Protons (H^+) constitute one of the major energy currencies of the plant cell, on a par with NAD(P)H and ATP. At the inner mitochondrial membrane and at the thylakoid membranes, a transmembrane H^+ potential is generated and used to energize the synthesis of ATP.

At all other membranes in the cell, H^+-pumps hydrolyze ATP to power the transport of protons out of the cytosol, establishing electrochemical potentials across

these membranes as well. The resulting transmembrane H^+ potentials are then used to power the transport of other ions and solutes across the membranes. Typically the cytosol pH is 7.5, the apoplastic pH is near 5.5, and the membrane potential difference is on the order of –150 mV. Under these particular conditions, the resulting pmf would be –268 mV. Values ranging from –200 to –300 mV are common in plant cells.

Active transport, results in the accumulation of a solute on one side of a membrane. It is thermodynamically unfavourable (endergonic), and occurs only when coupled (directly or indirectly) to an exergonic process such as the absorption of sunlight, an oxidation reaction, the breakdown of ATP or the concomitant flow of some other chemical species down its concentration gradient.

Carriers

Specific symporters and antiporters. These are transport systems that couple the down hill (exergonic) flow of ions such as H^+ or Na^+ to the uphill (endergonic) flow of inorganic ions and solutes are called carriers.

Transporters catalyzing solute flux in the same direction as H^+ or Na^+ flux are known as symporters. Carriers also undergo conformational changes during transport. There are no long-range interactions with soluble substrates and turnover rates are greater than those of pumps, typically being about 10^3 s^{-1}.

Excretion from the cytosol can be accomplished by antiporters, which exchange solutes for protons. Both symporters and antiporters tend to dissipate the pmf, and this energy is conserved in the form of an electrochemical potential for particular solutes.

Pumps

They undergo long-range and complex conformational transition(s) that extend across large polypeptides or assemblies of polypeptides. These movements couple the metabolic reactions to those involving transport. The turnover rate of pumps is 10^2 molecules transported per second. Pumps have slow turnover number and generate the pmf for an array of symporter and antiporters. A square micrometer of membrane may include several hundred to several thousand pump proteins, while it typically contains only 1 to 10 channel proteins. The rapid turnover rates and diffuse distribution of channels facilitate electrophysiological analysis of single channel molecules. The *proton pumps* at the vacuolar and plasma membranes are electrogenic. They create electrical current because the ions they remove from the cytosol carry charge. Therefore, this H^+-pumping ATPase not only contributes directly to the chemical

component of the pmf, the △pH, but also tends to make the electrical component, Vm more negative.

Ion channels for K^+, Ca^{2+} and anions

The net direction of ion flux through a channel is determined solely by the electrochemical force acting on that ion. Channels, in contrast to pumps and carriers, do not undergo conformational changes during transport and can catalyze ion fluxes of 10^6 to 10^8 s^{-1}. The rate of Na^+ movement through the acetylcholine receptor ion-channel is linear with respect to the extracellular Na^+ concentration; the process is not saturable in the way that transporter-catalyze.

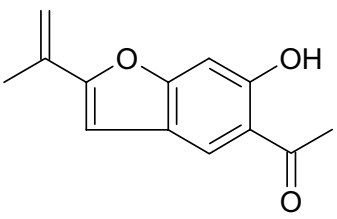
F-type H^+-pumping ATPases are found in plants at the inner mitochondria and *thylakoid* membranes that synthesize ATP

These two kinds of membranes contain proton-pumping electron transport chains driven by the redox potential and by light energy respectively. The pmf established by the electron transport chain serves to drive H^+ flow back through the F-type ATPase, thereby resulting in ATP synthesis. One sector of a F-type ATPase called F_0 in plant mitochondria and CF_0 in plant chloroplasts, transverses the membrane and forms an H^+-conduit. The other sector of the enzyme called F_1 in mitochondria and CF_1 in chloroplasts, readily dissociates from the transmembrane sector, contains adenine nucleotide-binding sites and can hydrolyze ATP in vitro. The flow of H^+ through F_0 drives long-range conformational transitions in F_1 that result in the synthesis of ATP. Crystallographic studies of the F_1 sector $\alpha_3\beta_3\gamma$ complex (located primarily on the β subunits), show three distinct conformations. At any given time, one binding site is in an open conformation, another is binding a nucleotide loosely, and the third is binding a nucleotide tightly, the conformational model for proton-driven ATP synthesis, which postulates that ATP is synthesized by F-type ATPase through a process of rotational catalysis (Lores Arnaiz, 1998). According to the conformational model, ADP and inorganic phosphate first bind to the open nucleotide-binding sites. Proton flow through the F_0 sector of the enzymes causes the central F_1 subunit (γ) to rotate, altering the conformation of all three nucleotide-binding sites. The tight binding site opens and releases ATP into the aqueous medium, the open site is converted to a loose site, and the loose binding site forms a tight pocket in which ATP is formed spontaneously. In net terms, a total of three or perhaps four protons are admitted through the F_0 sector for each ATP synthesized.

Energy transfer inhibitors

Here it was found that the F-Type H^+-pumping ATPase from chloroplast is a good target of interaction for allelochemicals, thus the following secondary metabolites Euparin 48, piquerol A 49 (Méndoza et al. 1994), dehidrocostus lactone 50, costunolide 51, 7-oxo-7-deacetoxygedunin 52, gedunin 53, and 5-O-β-D-galactopyranosyl-7-methoxy-3', 4'-dihydroxy-4-phenylcoumarin 31 behave as energy transfer inhibitors, that is, F-Type H^+-pumping ATPase inhibitors. All these compounds inhibited the Mg^{2+}-ATPase activity of the bound membrane thylakoids, as do energy transfer inhibitors. The target was located at CF_0 in the case of 52, 53 and 35, because the proton uptake was re-established as the concentration of the inhibitor was increased in the assay medium, when the assay was done in CF_1 washed thylakoids. In the case of the tetranortriterpenoid 52 it was found that the ketone group at C-7 was an important structural requirement for the inhibitory activity exerted on the enzyme H^+-ATPase, since 53 possessing a 7α-acetoxy group was a less potent inhibitor to photophosphorylation.

Comparison of the type of activity displayed by the limonoids 22, 23 and 24, revealed that the nature of the heterocyclic ring at C-17 determines the mechanism and the target of this type of compounds. In the case of gedunin type, which possesses a furan ring at C-17, was observed to behave as energy transfer inhibitor. However 23 and 24 possessing a ketal ring at the same position behave as Hill reaction inhibitors.



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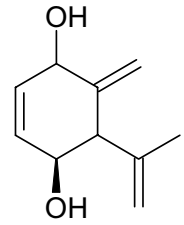

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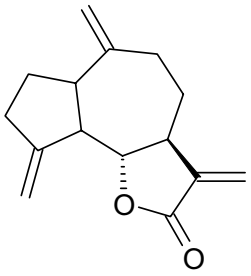
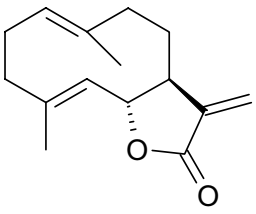
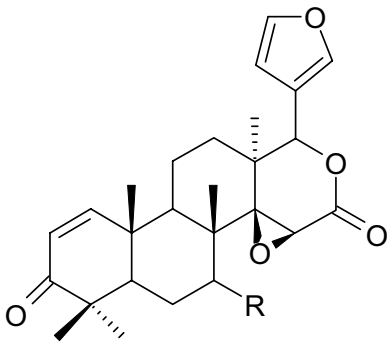




(50) **(51)**

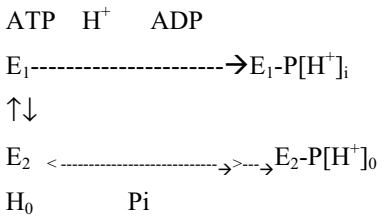


(52): R = O
(53): R = OAc

P-type ATPase, the plasma membrane H^+-pumping ATPase

It is a single polypeptide of about 100 KD that both binds ATP and catalyses H^+ transport, it is encoded by 10 genes in arabidopsis and tobacco. Isoforms of P-type H^+ ATPase appear to serve distinct function and may be expressed differentially in specific tissues and during particular phases of development.

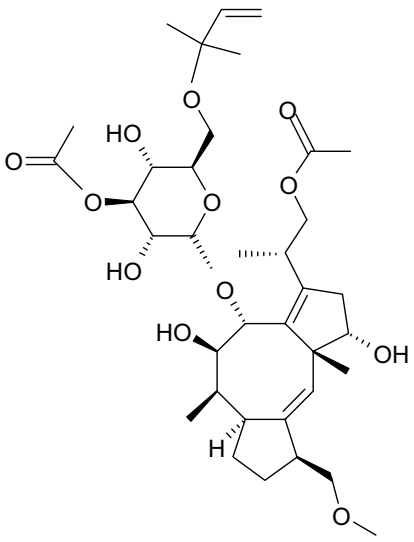
The pmf generated by the plasma membrane P-type ATPase powers transport through a variety of carriers; it also influences ion channel activity through its impact on Vm. The resulting acidification of the cell wall in turn activates expansins, proteins that loosen H^+-bonds within the wall and allow turgor-generated growth. It also produces an electrochemical gradient across the plasma membrane. Electrochemical gradient consists of an electrical potential (*inside negative*) and a concentration gradient (*a pH gradient outside acidic*). Many metabolic pathways result in the net production of H^+, some of which must be removed to prevent acidification of the cytosol. This is done by the plasma membrane H^+-ATPase. The plasma membrane ATPase pumps a single H^+ out of the cell for each MgATP hydrolyzed. During hydrolysis, the γ-phosphate of ATP becomes transiently but covalently bound to an aspartyl residue on the enzyme, forming an acyl-phosphate. Hydrolysis of this acyl-phosphate bond provides the driving force for the reaction cycle, in which different conformations of the enzyme, known as the E_1 and E_2 states, expose the H^+-binding site to alternate sides of the membrane.



The conformational change that exposes the bound H^+ to the apoplast is linked to additional conformational transitions that lower binding-site affinity, permitting H^+ dissociation.

This family of P-type enzymes includes the fungal plasma membrane H^+-ATPase, the Na^+/K^+-exchanging ATPase ubiquitous in animal cell plasma membranes, Ca^{2+}-ATPases of the plasma membranes of animals and plants, and the H^+/K^+-exchanging ATPase of mammalian gastric mucosa.

All P-type ATPases are inhibited by orthovanadate (H_2VO_4), which forms an analog of the E-P transition state and blocks the reaction cycle. In tomato and tobacco there are tissue specific H^+-ATPases, for example AHA3 is expressed selectively in phloem companion cells, the micropyles and the developing seed funiculus. In contrast, AHA10 is expressed mainly in developing seeds, especially in the tegument surrounding the embryo. From expression studies in yeast mutants that lack native plasma membrane H^+-ATPase activity, it is known that AHA1 and AHA2 have Km values for ATP of 0.15 mM, whereas the Km of AHA3 is 10-fold higher. Sensitivities to orthovanadate also differ. Regulation of plasma membrane H^+-ATPase is mainly an activation in response to lowered cytosolic pH. More profound perhaps, is the impact of the C-terminus cytosolic region of the enzyme, which forms an auto inhibitory domain and whose function has been identified by either tryptic cleavage or genetic modifications that activate the H^+-ATPase considerably. A striking twist to C-terminal H^+-ATPase inhibition has been observed with fusicoccin (54) (an inhibitor produced by *Fusicoccum amygdali*). Fusicoccin stimulates plasma membrane H^+-ATPase, in a whole range of plant cell types. Fusicoccin and trypsin-induced activation of the plasma membrane H^+-ATPase exhibit similar kinetic characteristics and are non additive, indicating that fusicoccin may activate the enzyme by relieving C-terminal auto inhibition. A separate mechanism of activation of H^+-ATPase is associated with auxin-induced stimulation of proton pumping. In this case, auxin up-regulates expression of the pump. Induction of H^+-ATPase expression correlates with the auxin-responsiveness of proton pumping in the tissue.



(54)

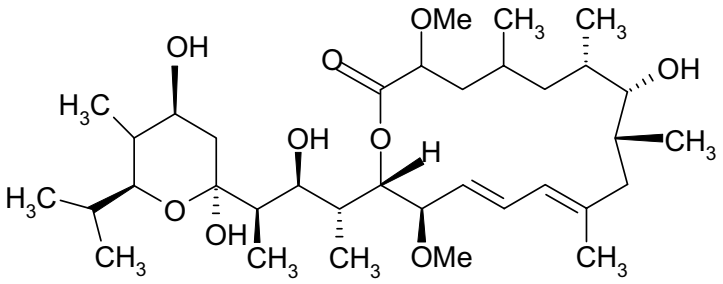
Calcium-pumping ATPases

They are distributed in the plasma membrane, the ER, the chloroplast envelope, and vacuolar membranes. These enzymes pump Ca^{2+} out of the cytosol, thereby maintaining the cytosolic concentration of free Ca^{2+} at about 0.2 μM. This low concentration of free Ca^{2+} has to be maintained in cells in order to prevent precipitation of phosphates. In eukaryotes it has become a base on which to build stimulus-response coupling pathways. All Ca^{2+}-ATPases are P-type ion-motive ATPases. Arabidopsis, has an ER-type Ca^{2+}-ATPase. In plasma membranes, Ca^{2+}-ATPase activity is enhanced by calmodulin. One of the Ca^{2+}-ATPases has been associated with the chloroplast inner envelope membrane; another resides in the vacuolar membrane. The vacuolar Ca^{2+}-ATPase has a calmodulin-binding domain, present as an extension at the N terminus, as for example the plant endomembrane "PM-type" Ca^{2+}-ATPases. The cytosol negative membrane potential opposes the export of divalent cations with double the effective force imposed on monovalent cations. The free energy of the Ca^{2+} electrochemical potential difference across the plasma membrane, roughly –60 KJmol-1, may actually exceed the free energy input available from ATP hydrolysis (-50 KJ mol-1). The enzyme also catalyzes Ca^{2+}/H^{+} exchange.

Vacuolar and other membranes are energized through vacuolar H^+-ATPases, a V-type H^+-ATPase

Plant vacuoles contain a highly acidic solution, with a pH near 5.5, about 2 to 3 units lower than that of the cytosol. Proton pumping into the vacuolar lumen not only energizes the membrane carrier-mediated transport but also generates the low pH of the vacuole, whereas protease, glucosidases, phosphatases and nucleotidases with acidic pH optima reside. Proton pumping is catalyzed by vacuolar type (V-type) H^+-ATPases. The sequence analysis has demonstrated that these enzymes are distant relatives of F-type H^+-ATPases. V-type ATPase operates solely in the direction of ATP hydrolysis. The ratio of H^+ translocated per ATP hydrolyzed has been measured as 2 or below when lumenal pH is low. The V-type enzyme can be separated into a soluble V_1 sector, analogues to F_1 and including the adenine nucleotide-binding sites, and a membrane-bound V_0 sector**,** analogous to F_0 and composing the H^+ pathway through the membrane. V-type H^+-ATPases is specifically inhibited by the macrolite antibiotic bafilomycin A_1 (55). It's a hydrophobic compound produced by Streptomyces and interacts with the V_0 sector of the enzyme. An acidic lumenal pH of all cellular organelles except mitochondria and chloroplast, contribute to vesicle sorting and hence to membrane trafficking and protein targeting. Therefore the V-Type H^+-ATPase could be a good candidate for allelochemicals interactions.

H^+-pumping inorganic pyrophosphatase (H^+-PPase) ubiquitous at the vacuolar membrane of plants. Could be also a good candidate for allelochemical interactions.



(55)

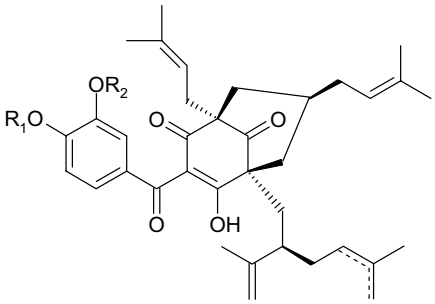
Tubulin/microtubule system

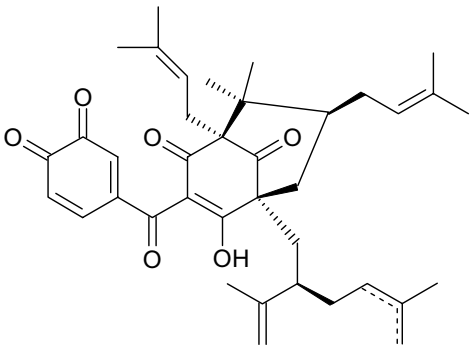
It is involved in many biological phenomena, such as formation of the mitotic spindle, constitution of the cell cytoskeleton, and axonal transport. Therefore, any substance able to interact with this system represents a potential inhibitor of cell replication. This is a promising target to search for anticancer agents from the plant kingdom.

Roux et al. (2000), have found the microtubule disassembly inhibitory properties of the frown polyisoprenylated benzophenones xanthochymol (56) and guttiferone E (57), isolated from the fruits of Garcina pyrifera . Furthermore, observation by electron microscopy of the microtubules assembled in the presence of mixture 1 (56+57) and cooled to 0ºC exhibited a classical pattern for microtubules; this last observation is contrary to that shown by paclitaxel, that in the same condition did not promote assembly of tubulin at 0ºC. The IC_{50} value was 2 μM for mixture 1. A strutucture-activity relationship study, found that etherification of the enol by methylation or cyclization led to a complete loss of activity on tubulin. The same is true if both hydroxyls at C-13 and C-14 are methylated or oxidized (58 and 59). However, some activity is preserved if only one of the hydroxyl groups at C-13 or C-14 is methylated (compound 60 and 61 cited in Roux et al. (2000), ethylated (62) or glycosylated (63). Hydrogenation of the double bonds (compound 64) also led to a total loss of activity. Therefore, the catechol and enol portions of the molecule are not the entire pharmacophore responsible for the biological activity, the lipophilic domain having the unsaturated prenyl chains is also essential because the octahydro derivative 64 is not active, although the catechol and enol parts are not modified.

However, the IC_{50} values for the cell growth inhibitor (for the cytotoxicity on KB cells) were similar for all the compounds studied. Therefore, it appears that cytotoxicity is probably not related to the interaction of the products with tubulin inside the cell. The tubulin/microtubule system assembly or disassembly is the preferred target for anticancer allelochemicals.

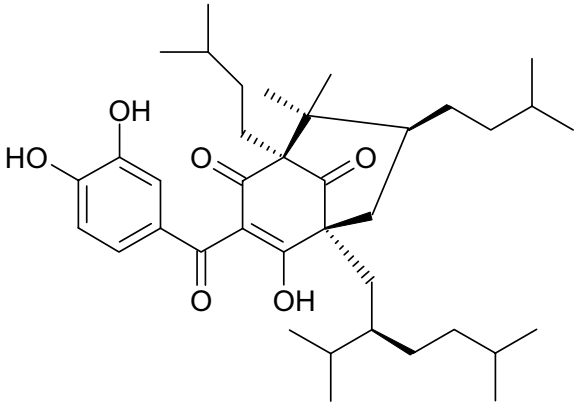
Xanthine oxidase (XO) catalyzes the formation of uric acid from the purines hypoxanthine and xanthine, and is responsible for the medical condition known as gout. Gout is caused by the deposition of uric acid in the joints leading to painful inflamation. Inhibiton of XO leads to a remission in gout (Chiang et al. 1994). XO also serves as an important biological source of oxygen-derived free radicals that contribute to oxidative damage to living tissues that are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Chiang et al. 1994; Cos et al. 1998). Therefore, in-vitro bioassays are used to examine test materials for XO inhibition, as inhibitors of XO may be potentially useful for the treatment of gout or other XO-induced diseases (Goodman and Gilman, 1990).





(56) : $R_1 = R_2 = H$, Δ^{36}
(57) : $R_1 = R_2 = H$, Δ^{35}
+(58) : $R_1 = R_2 = Me$
(60) : $R_1 = Me$, $R_2 = H$
(61) : $R_1 = H$, $R_2 = Me$
(62) : $R_1 = Et$, $R_2 = H$
(63) : $R_1 = \beta$-D-Glc, $R_2 = H$

(59)



(64)

Sweeney et al. (2001), studied extracts from Australians plants and assayed them for inhibition of the enzyme xanthine oxidase. The tree species of *Clerodendrum floribundum* R. Br., *Eremophila maculata* (Ker Gawler) F. Muell and *Stemodia grossa*

Benth all exhibited IC_{50} values less than 50 μg/mL. The most active plant species examined was *Clerodendrum floribundum* R. Br, extract E3 with an IC_{50} of 6.00 μg/mL. This active plant may contain bioactive natural products useful in the treatment of gout or other xanthine oxidase induced diseases. The target of these unknown allelochemicals seems to be XO.

SINGLET OXYGEN AND FREE RADICALS PROCESSES IN PLANTS

Singlet oxygen ($^1O_2$), a potential product of photochemical reactions of many allelochemicals and chloroplasts, is a damaging agent to all living organisms. How is the formation and control of $^1O_2$ within the plants? Does it possibly have a role in plant defense?

The ground state of molecular oxygen has two unpaired electrons with parallel spin. The triplet state is rare in ground state molecules and because electrons occupying the same orbital must have opposed spin (the Pauli exclusion principle), the reaction of ground state oxygen with most substances is restricted. The activation of oxygen involves overcoming this spin restriction to reactions. Reduction of $^1O_2$ leads to the potentially toxic superoxide anion, hydrogen peroxide and the hydroxyl radical. Electronic excitation of oxygen molecules, involving spin inversion, results in excited states with an unopposed spin, designated as singlet states $^1O_2$. Two excited singlet states of oxygen occur. However, the first singlet state ($^1\Delta g$) excitation energy of 0,98 eV, its lifetime is long enough to allow chemical reaction. Therefore, the first excited singlet state of molecular oxygen is the one involved in certain photooxidative, photodynamic and biological processes. The major mechanism of $^1O_2$ formation in biological systems is by energy transfer from photoexcited compounds. The absorption of a photon by such sensitizer results in an excited singlet state, with a very short lifetime (10^{-6} – 10^{-8} sec):

$$S \rightarrow {}^1S$$

which by intersystem crossing, involving spin inversion, may be relaxed to a longer lived triplet state (Ca 10^{-3} sec):

$$^1S \rightarrow {}^3S$$

Molecular oxygen in a non spin restricted reaction, can quench such a triplet state by energy transfer resulting in $^1O_2$ and the regeneration of the ground state sensitizer

$$^3S + {}^3O_2 \rightarrow S + {}^1O_2$$

The relatively low excitation energy of its first excited singlet state allows oxygen to quench the triplet states of a variety of compounds. Photosensitizing compounds, capable of the efficient population of triplet states, one of diverse origin and structure

and are active in regions of the electromagnetic spectrum ranging from the near UV, through visible to the near infra-red (Knox and Dodge, 1985). Singlet oxygen is responsible for type II photodynamic reactions of exogenous and endogenous sensitizes in biological systems, although direct reaction of the excited sensitizer with a substrate (a type I reaction) can occur (Knox and Dodge, 1985).

Singlet oxygen is a metastable entity of $^1O_2$ that varies from 2 to 4 μsec in H_2O to 25-100 μsec in non-polar solvents (cited in Knox and Dodge, 1985). Therefore, $^1O_2$ potentially is a damaging agent in membrane where it is generated by allelochemicals. Evidence has been presented indicating the increased lifetime of $^1O_2$ in the hydrophobic interior of the membrane relative to the aqueous environment of the cell. Although $^1O_2$ has no spin restriction to reaction, it is not indiscriminantly reactive. Its electrophilic nature results in chemical reaction with compounds with heavily substituted double bonds or an electron-rich functionality. In addition, $^1O_2$ may be physically quenched in its ground state by a variety of substances. As a consequence of this selectivity these are certain biomolecules that are cellular targets for action of $^1O_2$. Specific aminoacids, most notably histidine, methionine and tryptophan, are susceptible to oxidation by $^1O_2$ with the possible consequence of enzyme inactivation. Membrane destruction, a common feature of photodynamic reactions, is due to lipid peroxidation initiated by the formation of hydroperoxides from the reaction of $^1O_2$ with unsaturated fatty acids. Of the nucleic acid, guanine is particularly sensitive to attack by $^1O_2$. others classes of compounds may quench as well as react with $^1O_2$ for examples phenol, α-tocoferol (vit E), carotenoids and certain amines. Ascorbate acts as an effective quencher of superoxide (Knox and Dodge, 1985), hydroxyl radical and $^1O_2$. These compounds protect the cell for the $^1O_2$ damaging.

Singlet oxygen and secondary plant substances. Certain defensive allelochemicals are capable of photosensitizing reactions that involve the transfer of light energy to oxygen, such as $^1O_2$, which is used for their own defense. Other secondary plant products (Knox and Dodge, 1985) may have a physiological role in that they protect the plant against damaging photodynamic reactions by quenching the excited singlet state of oxygen. Here, some of the secondary metabolites involved in $^1O_2$ generations will be described.

QUINONES

The hypericins, occurring in the genus *Hypericum* (St John's Wort) are responsible for the photosensitization occurring when grazing animals ingest these plants. The condition of intense skin irritation is known as hypericism. Hypericum is contained in specialized trichome glands, presumably as a protection against auto toxicity, located on flowers, stems and leaves. Evidence has been presented that hypericin, isolated from the calyx of *Hypericum hirsutum*, is capable of the generation

of $^1O_2$ and hence lipid peroxidation. The photodynamic reactions of hypericin are promoted by visible light, predominantly in the region of 500-600 nm. Hypericin (65) has been shown to be phototoxic to the larvae of Aedes atropalpus mosquito. Fagopyrin (66), a hypericin derivative, occurs in the flowers of buck wheat (Fagopyrin esculentum), and gives rise to a photogenic condition in animal herbivores that is comparable to hypericin. Cercosporin (67) a fungal toxin, is produced by a member of the genus Cercospora, which includes fungi that are responsible for leaf spot diseases of a wide range of plants of economic importance. The photodynamic action of this toxin results in lipid peroxidation and membrane damage that resembles that of the pathogen. Evidence indicates that (67) is capable of photochemical generation of $^1O_2$ by this secondary metabolite.

Furanocoumarins (psoralen) (68), characteristic of the Rutaceae and Apiaceae, possess photosensitizing activity in mammalian skin. This photosensitizing activity arises from their ability to photobind to pyrimidine bases of DNA, resulting in crosslinks, when irradiated by long wavelength UV light [320-400 nm]; an activity that does not involve molecular oxygen. However, it is apparent that furanocoumarins are also capable of photodynamic reaction involving oxygen. They are found in all parts of the plants and are generally located in oil gland and ducts, seed coats and leaf surface wax.

Interestingly, angular furanocoumarins although capable of binding to DNA are unable to induce crosslink but do appear to be more efficient generators of $^1O_2$ than linear furanocoumarins.

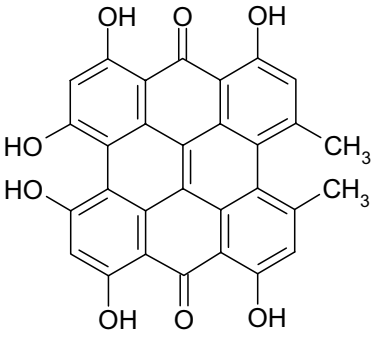
The polyacetylenes and their thiophene derivatives are a diverse class of compounds occurring predominantly in the Compositae. These compounds are also activated by near UV light (320-400 nm), but differ to furanocoumarins in that they are not capable of interacting with DNA. Polycetylenes are found in all parts of the plants including the cuticle but are frequently restricted to specific organs such as the roots.

Isolation of α-Terthienyl (69) is isolated from the roots of marginol (Tagetes), its nematocidal activity was demonstrated to be greatly enhanced by light and α-terthienyl is efficient generator of $^1O_2$ and the primary mechanism of action is the photodynamic disruption of membranes. Its phytotoxicity to microorganisms, insects and plants has been demonstrated. Therefore, maybe it is an allelopathic agent with an herbicidal potential.

Phenylheptatriyne (70) found in the leaves of *Bidens pilosa,* is the most studied polyacetylene, and also displays phototoxic action and antifeedant activity.

In contrast, photosensitization involving straight chain and ring-stabilized polyacetylenes such as (70) appears to be predominantly non-photodynamic under anaerobic conditions. The biological activity of these secondary metabolites against a

wide range of organisms suggests that they may have a protective role within the plant, especially against insect predators.



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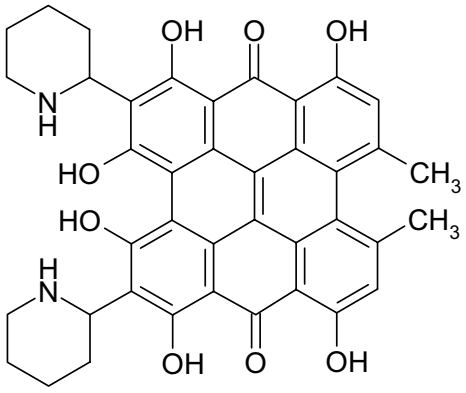

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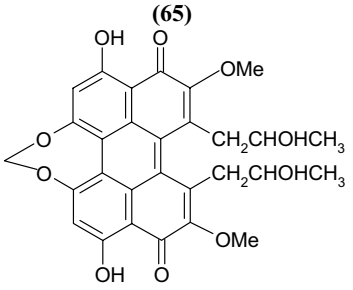

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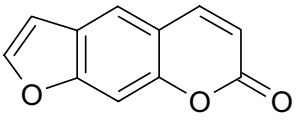

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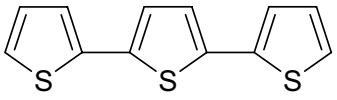

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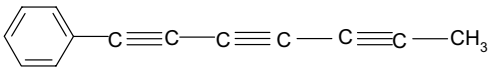
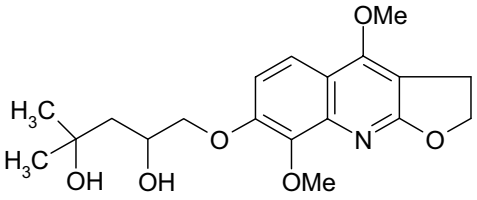


(70)

Furanoquinoline alkaloids such as dictamine (71) (Rutaceae) are phototoxic against microorganisms and mosquito larvae. Several β-carboline alkaloids such as harmaline (72) and isoquinoline (73) and alkaloids such as bereberine (74) are also phototoxic. Benzofurans and chromenes, from species of the genus Encelia are capable of yielding phototoxic reactions. Photoactivation by UV light of a range of isoflavonoid phytoalexins has been reported and appears to involve free radicals with

$^1O_2$. The production of $^1O_2$ during the autooxidation of tannins has been proposed to have a protective role by their fungistatic and deterrent effects.



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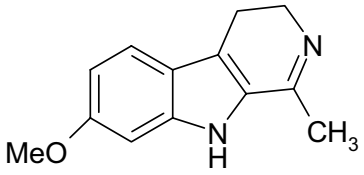

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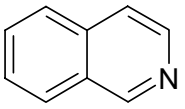

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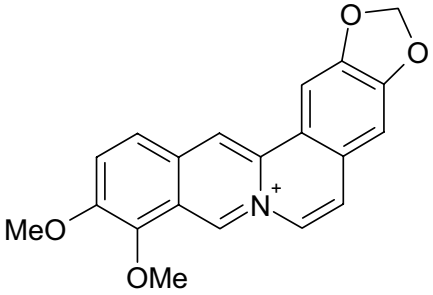

(74)

Secondary metabolites from plants of diverse biogenetic origin are capable of the photogeneration of $^1O_2$ suggesting the widespread use of this potent toxic agent as protective and defensive, and may be a potential herbicide. It is also possible that $^1O_2$ damage involves many plant stress conditions such as high light, limited water, presence of herbicides. The specificity of the allelochemicals that generate $^1O_2$ is required in order to avoid an unspecific phytogrowth toxicity.

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