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Biosynthesis of friedelane and quinonemethide triterpenoids is compartmentalized in *Maytenus aquifolium* and *Salacia campestris*☆

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Abstract

Maytenus aquifolium (Celastraceae) and *Salacia campestris* (Hippocrateaceae) species accumulate friedelane and quinonemethide triterpenoids in their leaves and root bark, respectively. Enzymatic extracts obtained from leaves displayed cyclase activity with conversion of the substrate oxidosqualene to the triterpenes, 3β-friedelanol and friedelin. In addition, administration of (±)5-^{3}H mevalonolactone in leaves of *M. aquifolium* seedlings produced radio labelled friedelin in the leaves, twigs and stems, while the root bark accumulated labelled maytenin and pristimerin. These experiments indicated that the triterpenes once biosynthesized in the leaves are translocated to the root bark and further transformed to the antitumoral quinonemethide triterpenoids.

Keywords: *Maytenus aquifolium*; Celastraceae; *Salacia campestris*; Hippocrateaceae; Friedelanes; Quinonemethides; Oxidosqualene; Cyclase activity; (±)5-^{3}H-mevalonolactone; Biosynthesis

1. Introduction

Quinonemethide triterpenoids are secondary metabolites restricted to the higher plant families, Celastraceae and Hippocrateaceae (Bruning and Wagner, 1978). These compounds have shown a variety of biological activity such as antitumoral (Bhatnagar et al., 1951; Gonçalves de Lima et al., 1971), antimicrobial (Ferreira de Santana et al., 1971), antibiotic (Bhatagar et al., 1951; Gonçalves de Lima et al., 1969a; Gonzalez et al., 1977), antimalarial (Pavanand et al., 1989) and spermicidal activities.

The co-occurrence of friedelanes and quinonemethides in several plants and in cell cultures has led to the postulation of a biogenetic relationship between these two classes of triterpenoids (Marini-Bettolo, 1979; Kutney et al., 1981; Gunaherath and Gunatilaka, 1983; Gunatilaka and Nanayakkara, 1984; Corsino et al., 1998) (Fig. 1). Nevertheless, no experimental work has been addressed to answer this question and thus the biosynthetic origin of the quinonemethide triterpenoids remains uncertain.

The working model under evaluation in this study is based on the compartmentalized biosynthesis and accumulation of friedelane derivatives and quinonemethides, respectively, in leaves and in root bark of Celastraceae and Hippocrateaceae species (Gonçalves de Lima et al., 1969b, 1972; Martin, 1973; Gonzalez et al., 1975; Martinod et al., 1976; Reddy et al., 1976; Dominguez et al., 1979; Furlan et al., 1990; Gamlath et al., 1990).

The entry point for biosynthesis of quinonemethides requires oxidosqualene as a central intermediate, which by the action of a cyclase would give rise to the first cyclic intermediate, 3β-friedelanol (Fig. 1) and then, by the action of an oxidoreductase, the conversion to friedelin should occur between leaves and root bark. The transformation/translocation steps for the

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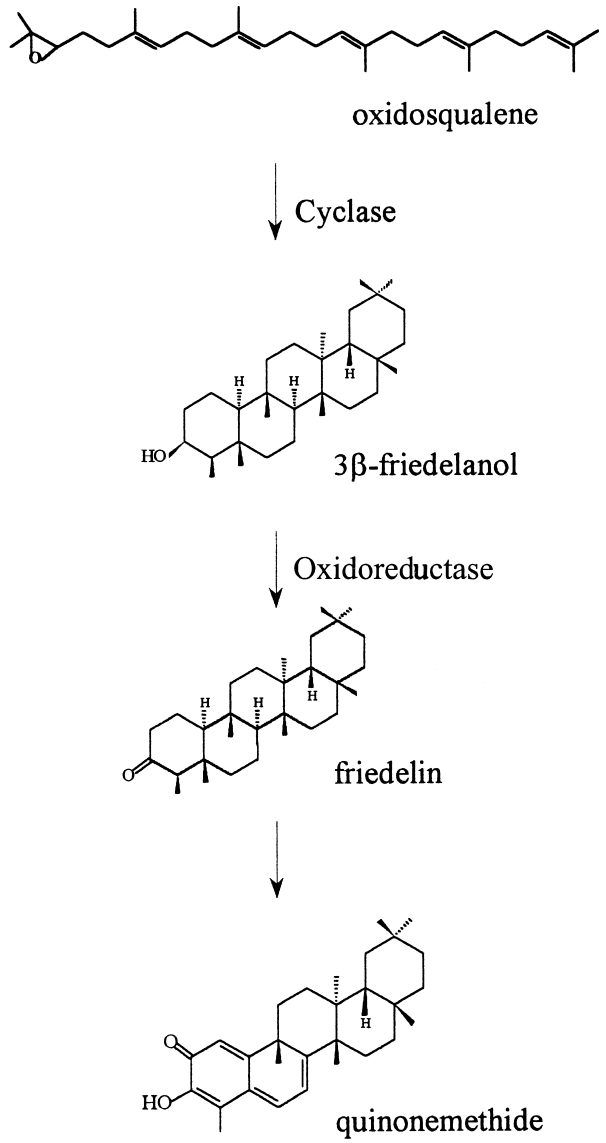


Fig. 1. Conversion of oxidosqualene to 3β-friedelanol and friedelin and their involvement as the biosynthetic precursor to the quinonemethides in *M. aquifolium* and *S. campestris*.

quinonemethide triterpenoids should take place in the root bark.

In order to evaluate the enzyme activity for the biosynthetic step involving the cyclase and its localization in leaves and/or root bark of *M. aquifolium* (Celastraceae) and *S. campestris* (Hippocrateaceae), we developed a protocol to solubilize and to determine the activity of this cyclase using oxidosqualene as substrate. If the quinonemethide and friedelane triterpenoids present in root bark and leaves, respectively, are continuously synthesized, then the corresponding tissue should display this cyclase activity.

Administration experiments with (±)5-^{3}H-mevalonolactone carried out in leaves of *M. aquifolium* seedlings were crucial in the determination of the translocation hypothesis of the friedelin from leaves to root bark, and thus the biosynthetic relationship with the quinonemethide triterpenoids. The results obtained are described below.

2. Results and discussion

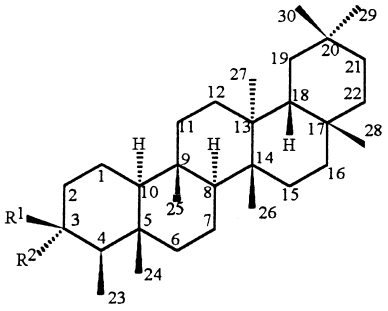
2.1. Friedelane and quinonemethide content

The friedelane and quinonemethide contents in leaves and root bark of *M. aquifolium* and *S. campestris* are shown in Table 1. Leaves of *M. aquifolium* and *S. campestris* yielded the highest friedelane derivative amounts, with 3β-friedelanol (**1**) and friedelin (**2**) accounting for more than one half of the triterpene content. As previously shown, the quinonemethides were not detected in leaves of either species. Nevertheless, in the root bark, the quinonemethides **3–8** were detected as the major components accounting for up to 0.2%. The extracts prepared from 3-year old *M. aquifolium* root bark yielded a high content of maytenin (**3**) (0.024%) and pristimerin (**4**) (0.097%) (Table 1). The root bark extracts of 7 year old plants of *S. campestris*, showed, in addition to **3** (0.32%) and **4** (0.033%), in increasing order of concentration 22β-hydroxymaytenin (**5**), 20α-hydroxymaytenin (**6**), celastrol (**7**) and netzahualcoyone (**8**) (Fig. 2).

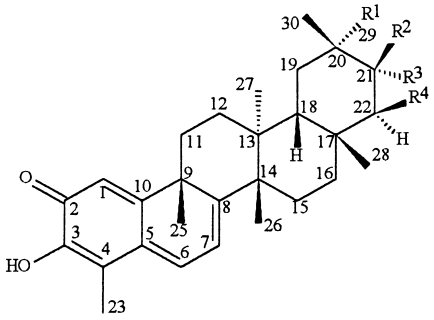
The pattern of triterpene distribution in these two species is similar to that previously reported (Gonçalves de Lima et al., 1972; Reddy et al., 1975; Martinod et al., 1976; Dominguez et al., 1979; Furlan et al., 1990) i.e. with the exclusive accumulation of friedelane derivatives in the leaves, whereas the quinonemethides are the major constituents in the root bark and are absent in leaves of both species.

Table 1
Content of friedelane and quinonemethide derivatives in leaves and root bark of *M. aquifolium* and *S. campestris* (contents are expressed as % of dry wt)

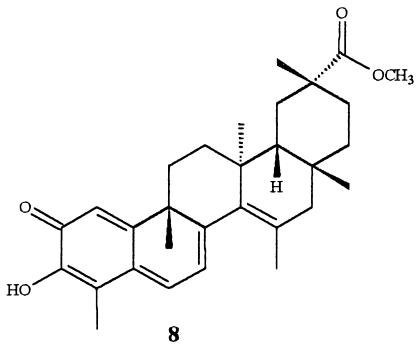
Plant tissue	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
M. aquifolium leaves	0.0088	0.0092	–	–	–	–	–	–
Root bark	–	–	0.024	0.097	–	–	–	–
S. campestris leaves	0.0094	0.0098	–	–	–	–	–	–
Root bark	–	–	0.032	0.033	0.013	0.022	0.021	0.052



1. R^1=OH, R^2=H
2. R^1=R^2=O



3. R^1=H, R^2=R^3=O, R^4=H
4. R^1=$COOCH_3$, R^2=R^3=H, R^4=H
5. R^1= H, R^2=R^3=O, R^4=OH,=H
6. R^1=OH, R^2=R^3=H, R^4=H
7. R^1=COOH, R^2=R^3=H, R^4=H



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2.2. Formation of triterpenoid products

Since the accumulation of friedelanes and quinonemethides is a clear indication of their compartmentalized biosynthesis in the leaves and roots of *M. aquifolium* and *S. campestris*, the presence or absence of cyclase activity in these tissues would provide direct evidence for this assumption. Thus a protocol for the determination of cyclase activity was developed, by examining the conversion of oxidosqualene as a substrate using a cell-free extract prepared from leaves and root bark of *M. aquifolium* and *S. campestris*. The in vitro conversion of oxidosqualene to the friedelane derivatives 3β-friedelanol (**1**) was monitored by a GC method (see Section 3). The GC analysis also revealed that the enzymatic extracts catalyzed the formation of friedelin (**2**) (Fig. 4A and B) (see Section 3). The identification of these compounds was carried out by examination of their retention times with those of authentic samples (Fig. 3), and also by GC/MS in which both friedelanes could be identified by the presence of molecular ions and other significant peaks. Under the assay conditions studied, the formation of **1** and **2** appeared to be linear until ca. 50 min and up to 1.2 mg protein/ml. The control experiments in the absence of the substrate, oxidosqualene, or protein extracts showed no triterpene formation. The cofactor requirement was next investigated using the following conversion assays: (i) 1 mM EDTA; (ii) dialysis (to remove divalent cations in the buffer); (iii) dialysis and readdition of 30 mM Mg^{2+} or 30 mM Mn^{2+} ions. It was clear from these results that a divalent cation was required for cyclase activity as Mg^{2+} stimulated the cyclase activity, whereas Mn^{2+} inhibited the reaction. (Hohn and Vanmiddlesworth, 1986; Cane and Pargellis, 1987; Dehal and Croteau, 1988; Croteau and Purkett, 1989). This cyclase activity in crude extracts could not be concentrated by $(NH_4)_2SO_4$ precipitation (40–80% saturation), because a great loss of activity was observed, showing this methodology not to be efficient for future purification of this cyclase.

Having established the authenticity of the enzymatic transformation, a time-course analysis was next carried out, this being linear over a 50 min time-frame and reaching a maximum level within ca. 2 h. Assays were also conducted at various pH values, ranging from pH 6.5 to 9.0 (see Section 3). A broad peak of activity, centered at pH 7.0 was observed for friedelane derivative biosynthesis, typical of terpene cyclases (Croteau, 1987; Croteau and Purkett, 1989). The temperature dependence of the reaction was also investigated, and a maximum activity at 40°C was observed.

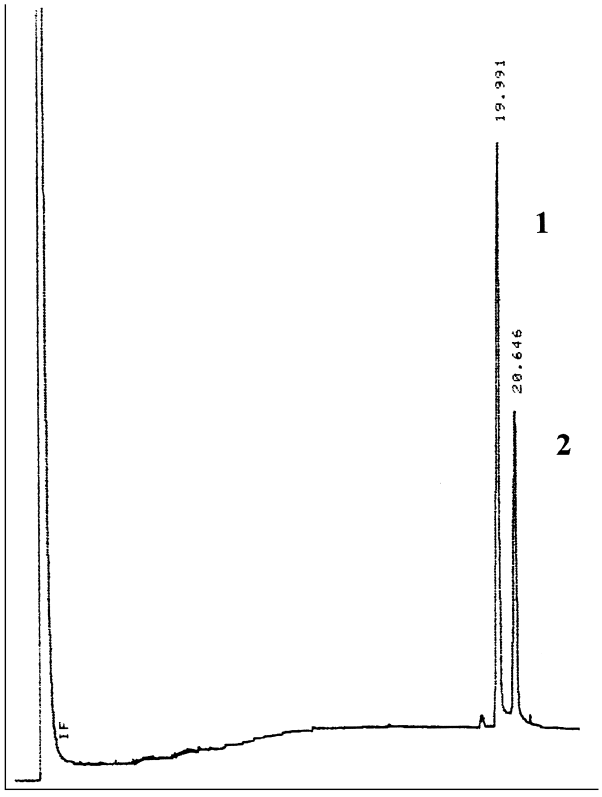

Fig. 3. GC chromatogram of the standards friedelan-3β-ol (**1**) and friedelin (**2**).

The cell-free extracts prepared from root bark of both species were incubated with oxidosqualene similarly but did not yield friedelane derivatives, thus this cyclase is present only in the leaves of these two species.

2.3. Possible sites for the biosynthesis of triterpenoids

Cell-free extracts prepared from leaves were capable of converting the oxidosqualene to 3β-friedelanol (**1**) and friedelin (**2**) as determined by GC (Fig. 4A and B). The percentages of conversion into 3β-friedelanol (**1**) and friedelin (**2**) were 0.020% (**1**) and 0.018% (**2**), respectively, in *S. campestris* whereas in *M. aquifolium* the conversion were 0.013% (**1**) and 0.012% (**2**). The control experiments indicated clearly that an enzyme was involved in this transformation as the boiled leaf extracts resulted in a major loss of activity. In conclusion, assays using protein extracts from leaves and root bark from Celastraceae and Hippocrateaceae species indicated the leaves as the possible site of biosynthesis of friedelane derivatives which should be further translocated to the roots and transformed to the quinonemethide triterpenoids. In order to confirm the possible translocation of friedelin derivatives from the leaves to root bark, a solution of (±)5-^{3}H-mevalonolactone was administered to the leaves of *M. aquifolium* seedlings (3 years old). Friedelin (**2**) was isolated from the leaves, twigs and stems after 3 days of incorporation, diluted with unlabelled carrier products followed by recrystallization (five times) to constant specific radioactivity. The results of the radioactivity measurements for friedelin (**2**) and quinonemethide triterpenes maytenin (**3**) and pristimerin (**4**) are given in Table 2. A dilution of relative specific activity for friedelin (**2**) was observed between leaves and stems. No labelled friedelin was detected in the root bark of *M. aquifolium* but, importantly, labelled quinonemethides maytenin and pristimerin could be observed.

These experiments including the determination of triterpenes and quinomemethides content, the feeding of (±)5-^{3}H-mevalonolactone associated with the determination of cyclase activity in cell-free extracts from leaves, twigs, stems and root bark of Celastraceae represents the first direct evidence to show compartmentalization of friedelane and quinonemethide triterpenoids.

3. Experimental

3.1. Plant material

Three year old seedlings of *M. aquifolium* were grown in the greenhouse of Departamento de Biotecnologia da UNAERP. The plants were harvested and immediately divided into root bark and leaves and frozen in liquid N_2. Leaves and root bark of *S. campestris* were collected in Fazenda Canchin at Universidade Federal de São Carlos (20 miles from Araraquara) frozen in liquid N_2, and immediately transported to Araraquara, where cell-free extracts were prepared.

3.2. Instrumentation

HPLC quantification was carried out as described by Corsino et al. (1998). Response factors were calculated with freshly prepared reference solutions of quinonemethides **3–8**. HPLC analyses were performed on a Shimadzu LC-10 AD Liquid Chromatograph using a reverse phase C_{18} column (150×4.6 mm i.d.) with a precolumn (20×4.6 mm i.d.) in the isocratic mode with eluent EtOAc:MeOH (95:05) at a flow of 1.0 ml/min. The column was purged with the mobile phase for 5 min, followed by equilibration for 4 h: the total analytical run time was 30 min. The spectral data were collected using a refraction index detector. The characteristic retention times for compounds **1** and **2** under these HPLC conditions were 23.26 and 24.03 min. GC analyses were performed on a Hewlett–Packard 5890 Series II Gas Chromatography with a split injector at 270° and a flame ionization detector (FID) at 310°. The injection volume was 2 μl and the split ratio was 1:10. Nitrogen was employed as carrier gas. A 30 m×0.25 μm SPB-5 capillary column was employed with temperature

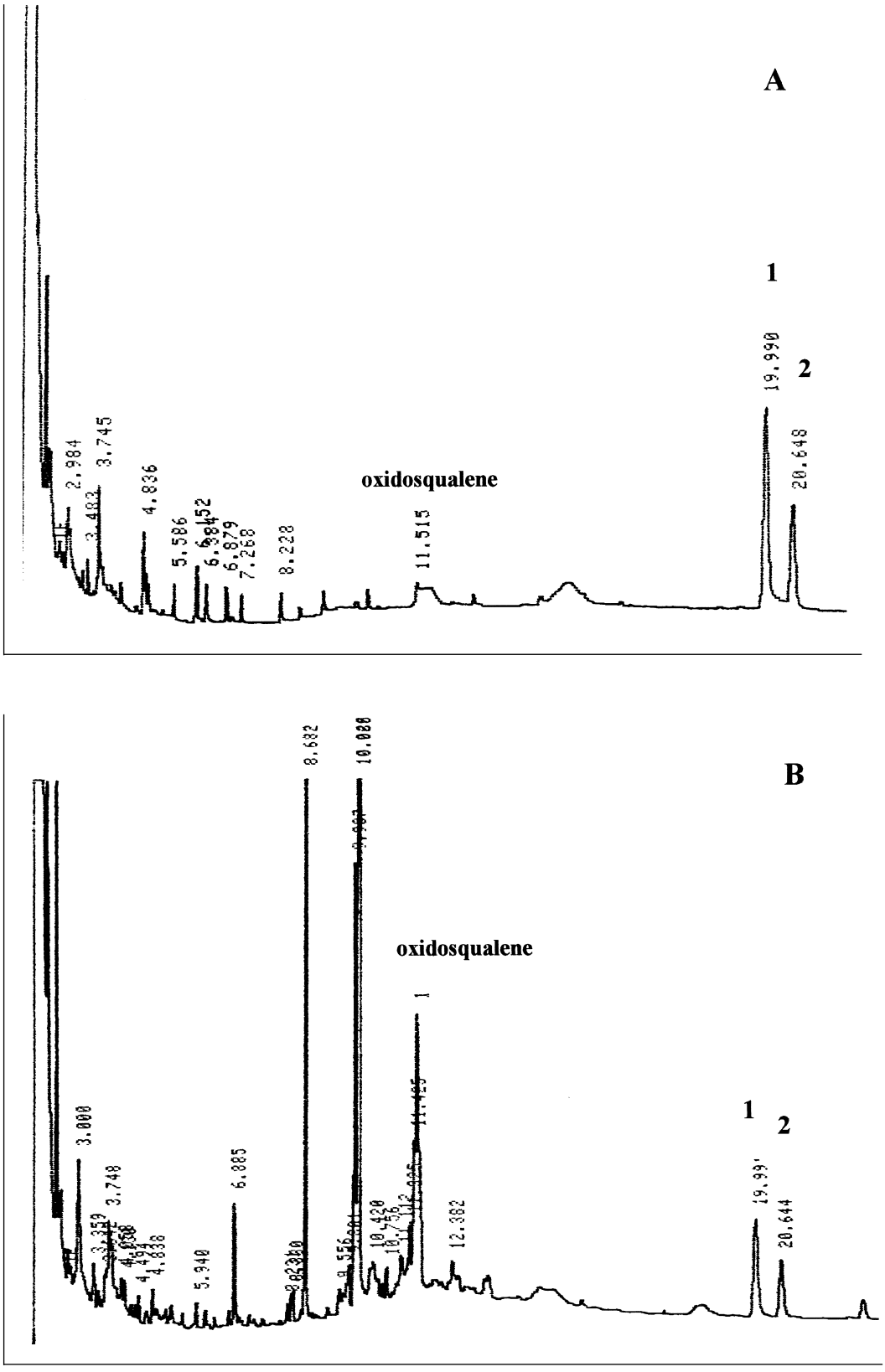

Fig. 4. Chromatographic profile-obtained from assays of cyclase activities in a protein extract from leaves of *S. campestris* (A) and *M. aquifolium* (B), respectively, using oxidosqualene as a substrate (for chromatographic conditions, see Section 3).

Table 2
Specific activities of friedelin (**2**), maytenin (**3**) and pristimerin (**4**)

Compound and tissue	Specific activity (dpm/mmol)	Relative specific activity (%)
*Friedelin (**2**)*		
Leaves	15.5×10^5	100
Twigs	10.1×10^5	65.2
Stems	9.0×10^5	58.1
*Maytenin (**3**)*		
Root bark	7.0×10^5	45.2
*Pristimerin (**4**)*		
Root bark	10.9×10^5	70.3

programming from 180° for 2.0 min, then increased by 5°/min to 280°, and maintained for 25 min. The data were collected with an HP 3396A integrator. GC/MS analyses were performed on a Hewlett–Packard 5970B. The injection volume was 2 μl and the split ratio was 1:10. Nitrogen was employed as carrier gas. A 30 m×0.25 μm HP-1 capillary column was employed with temperature programming from 50° for 2.0 min, then increased by 15°/min to 290°, and maintained for 25 min. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer at 200 and 50 MHz, respectively, using $CDCl_3$ as a solvent and TMS as reference. ES-MS were recorded on a VG Platform II spectrometer. Radioactivity was measured by liquid scintillation counting. (±)5-^{3}H-mevalonolactone (33mCi/mmol) was purchased from Sigma.

3.3. Friedelane and quinonemethide triterpenoids analysis

Root bark and leaves of *M. aquifolium* and *S. campestris* were dried for 24 h at 40° in an oven with forced air circulation. The dried and powdered leaves of *M. aquifolium* (4500 g) and *S. campestris* (3200 g) obtained were exhaustively extracted with ethanol. The resulting ethanol extracts were filtered and concentrated in vacuo to afford green gummy residues, 896.7 and 687.1 g, for *M. aquifolium* and *S. campestris*, respectively. The hexane soluble part of the ethanol extract *M. aquifolium* (96.5 g) and *S. campestris* (45.0 g) were applied to a Si-gel column (70–230 Mesh), eluted with hexane and increasing amounts of EtOAc (up to 70%) to give 55 and 35 fractions, respectively. Fraction 9 (0.175 g) of *M. aquifolium* and 7 (0.186 g) of *S. campestris* were subjected to Si gel column (230–400 mesh) chromatography, this being eluted with hexane and increasing amounts of EtOAc to give 3β-friedelanol **1** (0.043 and 0.074 g, respectively) and friedelin **2** (0.052 and 0.089 g, respectively). The structures of the compounds **1** and **2** were elucidated by comparison of their ^{1}H NMR, ^{13}C NMR and ES-MS data with literature values (Mahato and Kundu, 1994). The isolated and characterized compounds obtained were used as standards. The dried and powdered root bark of *S. campestris* (485 g) was extracted with CH_2Cl_2. The resulting CH_2Cl_2 extract was filtered and concentrated in vacuo to afford a red gum (100.9 g). This extract was submitted to filtration chromatography on Si gel (300 g) eluted with hexane–EtOAc (1:1), EtOAc and EtOAc–MeOH (1:1) and MeOH. The hexane–EtOAc (1:1) (11.6 g) fraction was subjected to silica-gel column chromatography, eluted with hexane containing increasing amount of EtOAc to give 45 fractions. Fraction 6 (0.296 g) was applied to preparative TLC, eluted with hexane–EtOAc–HAc (98:02:1%) yielding the fraction 6–3 (0.093 g) which was further purified by prep. TLC, eluted with hexane–EtOAc–HAc (90:10:1%) to give **3** (0.030 g), **7** (0.026 g) and **8** (0.046 g). Fraction 31 was subjected to preparative TLC, eluted with $CHCl_3$–MeOH–acetic acid (99:01:1%) to give **5** (0.007 g), **4** (0.012 g) and **6** (0.043 g).

The dried and powdered root bark of 3 year old *M. aquifolium* seedlings (20.24 g) was extracted with CH_2Cl_2. The resulting CH_2Cl_2 extract was filtered and concentrated in vacuo to afford a red gum (0.52 g). This extract was submitted to silica gel column chromatography (100 g) eluted with hexane with increasing amount of EtOAc to give 48 fractions. Fraction 35 (0.183 g) was applied to preparative TLC, eluted with hexane–EtOAc (4:1) to yield **3** (0.021 g) and **4** (0.053 g). The structures of compounds **3–8** were elucidated by comparison of their ^{1}H NMR, ^{13}C NMR and ES-MS data with literature values (Gunatilaka, 1996).

3.4. Protein extraction from leaves and root bark of *M. aquifolium* and *S. campestris*

All steps were carried out at 4°. Frozen leaves and roots (100 g) were ground in liquid N_2, using a mortar and pestle in the presence of XAD-4, and 30% (w/w) PVPP, 75 mM MES buffer, pH 7.0, containing 30 mM $MgCl_2$ or $MnCl_2$, 5 mM dithiothreitol (DTT), 20% (v/v) glycerol, 10 mM $Na_2S_2O_4$, 5 mM Na_2HPO_4 and 10 mM sodium ascorbate. The homogenate was stirred at 4°/60 min, filtered through Miracloth (Calbiochem) with the filtrate centrifuged (30 000 g, 20 min) to provide a supernatant which was used as the enzyme source.

3.5. Incubation of oxidosqualene with *M. aquifolium* and *S. campestris* leaves and root bark enzymatic extracts

The protocol for cyclase activity required protein extracts (190 μl) of leaves and root bark of the *M. aquifolium* and *S. campestris*, 50 μl of oxidosqualene (30 mM), and MES buffer (750 μl), pH 7.0 at 4°. Five assays were conducted in parallel. After 2 h incubation at 40° with shaking, the reaction mixture was extracted with EtOAc (300 μl). Thereafter, the EtOAc layer was evaporated to dryness and analysed by GC. For determining cofactor requirements, 10 μl of a 30 mM

solution of $MgCl_2$ and $MnCl_2$ were individually added to the assay mixture.

3.6. Protein assay

Protein concentrations were determined according to Hartree (1972), using bovine serum albumin as the standard.

3.7. Chemical syntheses

3.7.1. Epoxidation of squalene

A solution of squalene (0.858 g, 2.09 mmol) in CH_2Cl_2 (6 ml), saturated aqueous sodium carbonate (6 ml) and *m*CPBA (0.719 g, 4.18 mmol) was stirred at 4° for 4 h. After the reaction was complete, as indicated by TLC, H_2O (4 ml) was were added, followed by solid Na_2SO_4 (0.100 g). To the reaction mixture was added Et_2O (3×20 ml), with the organic layer separated, washed successively with saturated aqueous $NaHCO_3$, saturated aqueous NaCl and H_2O (3×10 ml, each). The organic layer was dried (Na_2SO_4) and evaporated to dryness to yield a residue (0.092 g), which was purified by silica gel column (150 g) chromatography, eluted with hexane: EtOAc (95:05) to furnish the oxidosqualene (0.061 g, 68.5% of yield). The oxidosqualene obtained was submitted to gas chromatography/mass spectrometry analysis and the molecular ion at m/z 476, corresponding to the molecular formula of the oxidosqualene was monitored and compared with authentic sample data.

3.8. pH and temperature optimum

Standard assay conditions were used to determine the pH optimum of the cyclase, except that the buffer was either MES–NaOH (0.1 M, pH 5.0–7.0), Tris–HCl (0.1 M, pH 7.0–9.0). Temperatures between 0° and 60° were evaluated for the capacity to promote 3β-friedelanol **1** and friedelin **2** formation under the standard enzymatic assay.

3.9. Feeding experiment

(±)5-^{3}H-mevalonolactone in an ethanol solution (100 Ci) was evaporated, dissolved in methyl cellosolve and the solution painted on intact leaves of *M. aquifolium* seedlings (3 years old). After 3 days, the leaves, twigs, stems and root bark were harvested and extracted with dichloromethane. The extracts on concentration deposited triterpenoid derivatives which were further processed for the isolation of the friedelin derivatives and/or quinonemethide triterpenoids as described above. The radioactive compounds were diluted with unlabelled carrier (ca. 50 mg) and recrystallized (five times) to constant sp. act. (Table 2). The identities of the products were determined by both HPLC and analysis of the ^{1}H NMR spectra, these being compared to authentic standards.

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