CONFERENCE ON ISOPRENOIDS 2003
LATE PAPERS

These contributions were submitted so late after the deadline we were unable to include them into the indexed collection of abstracts. However, we did our best to let them appear despite the fact that the format, even after many editorial corrections, did not comply with the rules of the journal. We assume that sometimes the communication is more important than some formalities.

Editors

THE ORIGIN AND ACTIVITY OF ISOPRENOIDS IN PINES AND PINE BARK BEETLES

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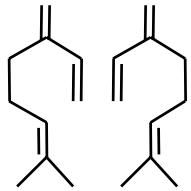
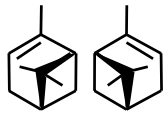
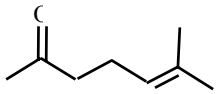
Pine-feeding bark beetles (Coleoptera: Scolytidae) interact with their host pines (Coniferales: Pinaceae) in the synthesis of one class of isoprenoids, the monoterpenoid aggregation pheromones. These pheromones are used to signal the mass attack of the beetles on pines, allowing the insects to coordinate feeding and mating in time and space. Examples of well-studied monoterpenoid pheromone components include ipsenol (**II**), ipsdienol (**III**), *cis*- and *trans*-verbenol (**V**, **VI**), and frontalin (**VIII**). Myrcene (**I**) is an acyclic pine oleoresin monoterpene that has been linked to the biosyntheses of ipsenol and ipsdieanol[1]; whereas α-pinene (**IV**) is a bicyclic pine oleoresin monoterpene that has been linked to the biosyntheses of *cis*- and *trans*-verbenol[2]. There is no obvious host monoterpene precursor for frontalin, but some have noted the presence of the geraniol derivative sulcatone (**VII**) in symbiotic fungi of bark beetles[3] and speculated that sulcatone may be an exogenous precursor to frontalin[4,5].



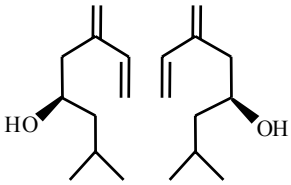
I. Myrcene



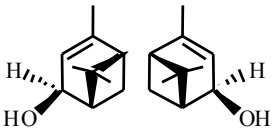
IV α-Pinene



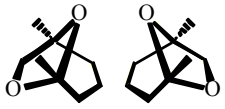
VII Sulcatone
6-methyl-5-hepten-2-one



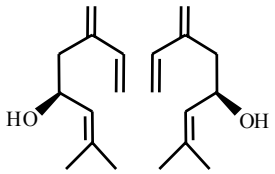
II Ipsenol
2-Methyl-6-methylene-7--octen-4-ol



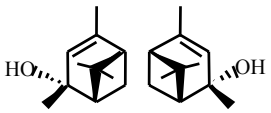
V *cis*-Verbenol
cis-4,6,6-trimethylbi-cyclo[3.1.1]hept-3-en-2-ol



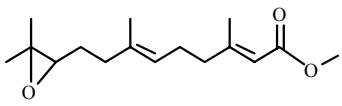
VIII Frontalin
1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane



III Ipsdienol
2-Methyl-6-methylene-
-2,7-octadien-4-ol



VI *trans*-Verbenol
trans-4,6,6-trimethylbi-
cyclo[3.1.1]hept-3-en-2-ol



IX Juvenile Hormone III
(±)-cis-10,11-epoxy-
3,7,11-trimethyl-E,E-2,6-
dodecadienoic acid
methyl ester

Fig. 1. Structures of isoprenoids in pines and pine bark beetles: pine monoterpenes, exogenous pheromone precursors, bark beetle aggregation pheromone components, and a bark beetle hormone.

A related structure, 6-methyl-6-hepten-2-one, has not been found in any hosts or symbionts of bark beetles, but may serve as the actual precursor to frontalin[6,7].

Research over the last 10 years has demonstrated that bark beetle pheromones can also be synthesized *de novo* (reviewed in[8]), with radiolabeling studies providing definitive proof for ipsenol and ipsdienol[9] and frontalin[10]. This endogenous synthesis occurs from acetate and mevalonate and is a highly regulated process that is stimulated at the transcriptional level by the sesquiterpenoid hormone, juvenile hormone III (JH III) (**IX**) (ref.[11-14]). In the case of *Ips* spp., the *de novo* biosynthesis of ipsenol and ipsdienol is evoked by feeding on the fresh phloem of the newly colonized host tree[11,15]; whereas in the case of the Jeffrey pine beetle, *Dendroctonus jeffreyi*, the *de novo* synthesis of frontalin appears to be initiated by emergence from the deteriorating phloem of the brood tree[16]. Whether *cis*- and *trans*-verbenol can be synthesized *de novo* by bark beetles remains an open question. There is some evidence from the mountain pine beetle, *Dendroctonus ponderosae*[17] and from *D. jeffreyi*[16] that the production of *trans*-verbenol by females can be stimulated by JH III.

Ultimately, the source of all carbon for pheromone production in bark beetles is from the food (i.e. pine phloem) ingested during the larval and adult stages[17]. However, we have only a rudimentary understanding of the degree of partitioning of bark beetle pheromone production between substrates originating from the host pine immediately prior to or during synthesis and substrates originating from the endogenous metabolic pool. To elucidate this biochemical insect-plant interaction requires focus on the late stages of the biosynthetic pathway (i.e. those reactions from isopentenyl diphosphate to the end products). Very recent work with the pine engraver, *Ips pini*, has demonstrated quite surprisingly that cell-free extracts of male tissue will convert geranyl diphosphate to myrcene[18]. This is the first evidence for a monoterpene synthase in the Metazoa and presents exciting new questions about the origin, evolution, and occurrence of terpene synthases in conifers and insects. This bark beetle monoterpene synthase is not only sex-specific, but its activity can be induced by prior treatment with JH III or by feeding on phloem from two host trees, Jeffrey pine, *Pinus jeffreyi*, and red pine, *Pinus resinosa*. The sex specificity and endocrine induction of this activity present a logical linkage of myrcene production in *I. pini* with pheromone biosynthesis. Ipsdienol is the principal pheromone component of *I. pini*[19], and this monoterpene alcohol can be synthesized from myrcene in this species[20].

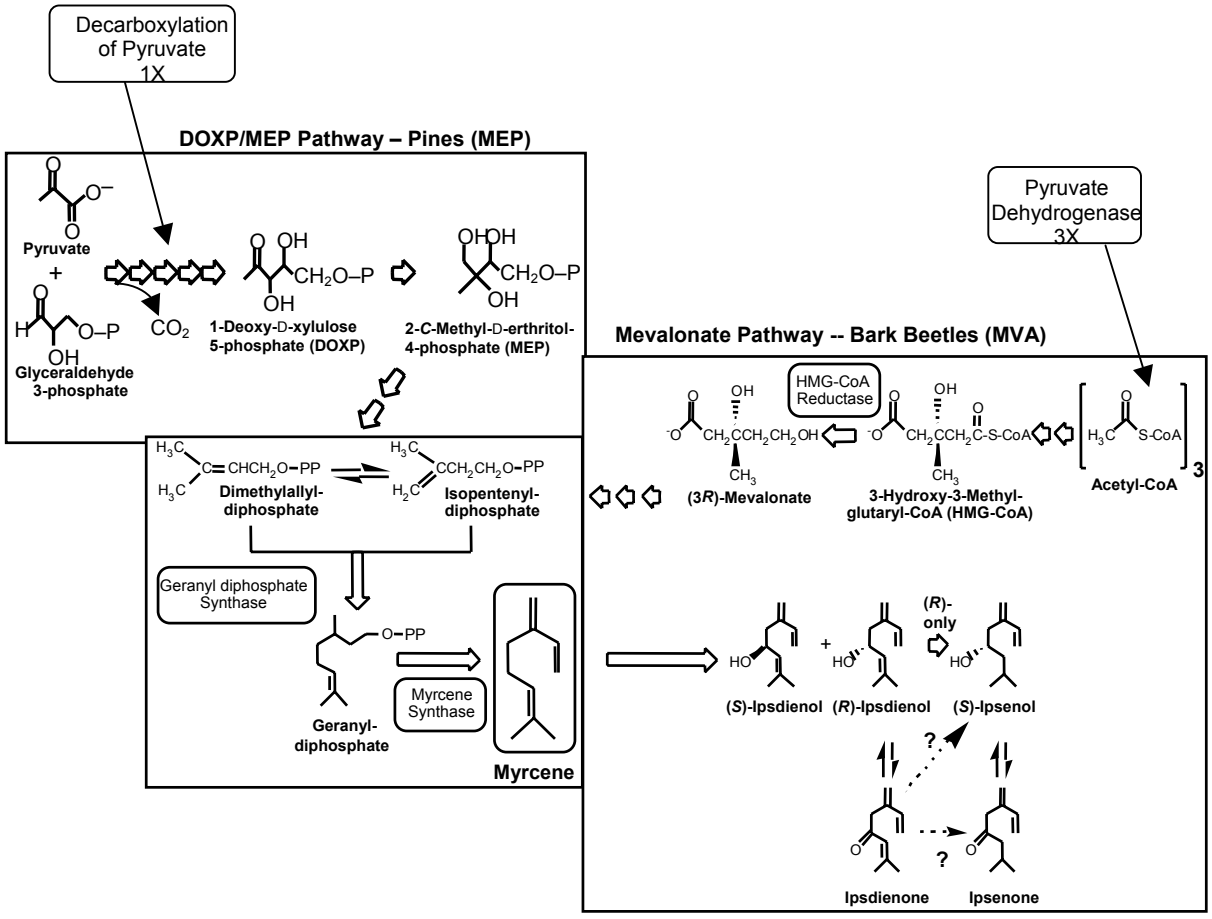
To further investigate the relationship between pine monoterpenes and bark beetle monoterpenoid pheromones, we have designed an experiment to compare the biosynthetic origins of myrcene and α-pinene from three species of pines with the biosynthetic origins of ipsenol, ipsdienol, *cis*- and *trans*-verbenol from three species of pine bark beetles. The pine engraver, *Ips pini*, the California fivespined ips, *I. paraconfusus*, and the pinyon ips, *I. confusus* (all bark beetles) were collected from Jeffrey pine, *Pinus jeffreyi*, ponderosa pine, *Pinus ponderosa*, and singleleaf pinyon pine, *Pinus monophylla*, respectively. These insects were collected from infested fallen stems and branches in the forests of the Sierra Nevada Mountains of California (*I. pini* and *I. paraconfusus*) or from infested standing trees in the forests of the Pinenut Mountains of Nevada (*I. confusus*). Concomitant with the collection of the insects, logs were collected from freshly cut live trees of the host pines. Newly emerged adult insects were reared from the infested pine logs, separated by sex, and stored temporarily at 4 °C on moist paper toweling.

Terpenoid-laden volatiles were trapped on Porapak Q (Supelco, Inc., Bellefonte, Pennsylvania, USA) by placing the freshly cut logs with and without artificially introduced *Ips* spp. into 19 l. glass carboys and drawing air for 7 to 10 days through the Porapak Q using published methods[19]. Samples were prepared with pheromone-producing males in the appropriate host logs (replicated 4 times), with non-pheromone producing females in the appropriate host logs (control, replicated once), and with only the host logs (control, replicated once). The 18 Porapak samples were extracted with pentane and the extracts will be analyzed by GC-FID, GC-MS, and isotope-ratio mass spectrometry (IRMS). Given comparative standard materials, this latter technique is a tool to determine the origin and history of organic compounds[21,22].

Hypothetically, the syntheses of monoterpenes by pines should occur in the plastids via the methylerythritol phosphate (MEP) pathway, whereas the monoterpene alcohols produced *de novo* by bark beetles should occur in the anterior midgut via the mevalonate (MVA) pathway. These two pathways, which

converge on isopentenyl diphosphate (Scheme 1), differ in the frequency with which the enzyme pyruvate dehydrogenase catalyzes the production of acetate from pyruvate. In the MEP pathway, the enzyme catalyzes one reaction as a prelude to the formation of 5-deoxy-D-xylulose-5-phosphate; whereas in the MVA pathway, mevalonate originates from three molecules of acetyl CoA (i.e. three catalytic events). As this thiamine-dependent pyruvate dehydrogenase prefers lighter isotopomers of pyruvate, i.e. those depleted in ^{13}C, a product of the MVA pathway should be relatively more depleted in ^{13}C isotopomers than a product of the MEP pathway. This difference in the endproducts of the MEP and MVA pathways can be measured using IRMS as the $\delta(^{13}C)$ value, which is expressed as $\delta(^{13}C)$ $[‰] = [(R_{sample}/R_{standard}) -1] \times 10^3$. *R* corresponds to the $^{13}C/^{12}C$ ratio of the sample and the standard (Vienna Pee Dee Belemite)[23]. The impact of the oxidation reactions that produce the monoterpene alcohols on the $\delta(^{13}C)$ value is not known.

The $\delta(^{13}C)$ values of monoterpenes have been reported in the literature to range from –26 to –30 ‰ (ref.[23]). Accordingly, preliminary results with the samples from *I. pini* show $\delta(^{13}C)$-values for myrcene of –27.1 ‰ (from volatiles collected from a log of the host tree, *P. jeffreyi*) and –27.5 ‰ (from volatiles collected from a log of *P. jeffreyi* containing male *I. pini*). The $\delta(^{13}C)$ value for ipsdienol from the same collection of volatiles above the headspace of feeding male *I. pini* was –25.1 ‰, which suggests that the oxidation reactions may dramatically increase the abundance of ^{13}C in the pheromone end product (even though it is synthesized via the MVA pathway). Results from the remainder of this study are pending analysis.



Scheme 1. Proposed interaction of monoterpenoid biosynthetic pathways in pines and bark beetles showing hypothetical alternatives for incorporation of ^{13}C resulting from decarboxylation of pyruvate in the MEP and MVA pathways[18].

METHODS

Coupled gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was conducted using a Finnigan MAT 252 mass spectrometer equipped with a ThermoFinnigan Trace GC gas chromatograph and CuO/Ni/Pt combustion furnace operated at 940 °C. The samples were injected splitless (0.8 min) onto a 30 m fused silica column (DB5-MS, 0.32 mm i.d., 0.25 µm film thickness). Injector temperature was 220 °C. Carrier gas: He. GC temperature program: 3 min 45 °C; 45 °C to 220 °C at 3 °C min^{-1} ; 10 min

220 °C. Standard deviations for replicate injection (3) varied from 0.1 to 0.5 ‰ and were typically less than 0.2 ‰. The stable carbon isotope compositions are reported in the δ notation against the VPDB ^{13}C-Standard.

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FUNGAL TRANSFORMATION OF SOME TERPENES AND STEROIDS

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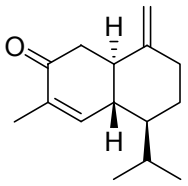
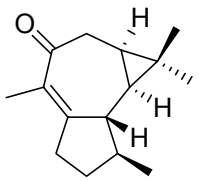
Jamaica is geographically situated in the centre of the Caribbean. The island boasts a number of micro-climates and this is reflected in the wide diversity of plant and microbial life. There are over 3,000 recorded flowering plants; a quarter of which are endemic[1]. Phytochemical analysis of some members of three plant families has been performed. Some of the biologically active terpenes isolated have been incubated with fungi in the hope of generating a series of analogues. Furthermore, for the first time, two locally isolated fungi have been examined for their potential for steroid transformation.

Hyptis verticillata, an example from the Labiatae, is used in traditional medicine to treat bronchial disorders. A number of terpenes (e.g. **I**, **II**)[2,3], flavonoids and lignans[4-6] have been isolated from this aromatic herb. Compound I has been shown to reduce the fertility of the cattle tick, *Boophilus microplus*, while both **I** and **II** are toxic to the sweet potato weevil, *Cylas formicarius elegantulus*[2,3].



I

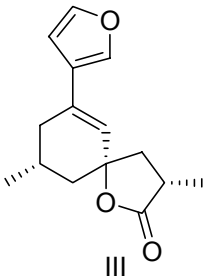
I – cadinane



II

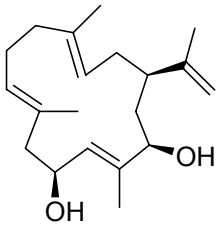
II - aromadendrane

Capraria biflora, a member of the Scrophulariaceae family, has been used in local folklore for various bacterial and viral infections. Although the extracts contain a large number of compounds, careful chromatographic separation has yielded four sesquiterpenes (e.g. **III**). Such caprariolides, as they have been called, possess a novel skeleton and have been found to exhibit insecticidal activity[7].



III

III - caprariolide A

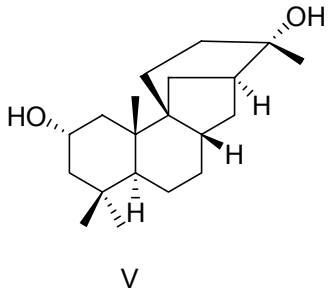


IV

IV - cembrane derivative

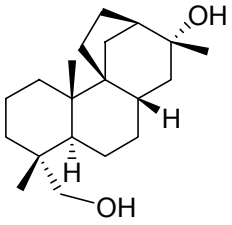
Cleome spinosa, from the Capparaceae, yields large amounts of a new cembrane (**IV**) along with four congeners[8]. Terpenes with the cembrane skeleton are known to possess

diverse biological activity including potent cytotoxicity against a number of human cancer cell lines, inclusive of leukaemia, melanoma, breast and colon carcinomas.



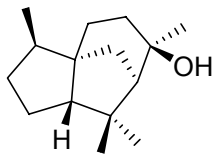
V

V - stemodin



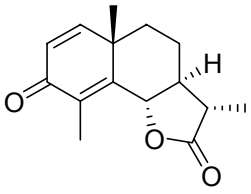
VI

VI - stemarin



VII

VII - cedrol

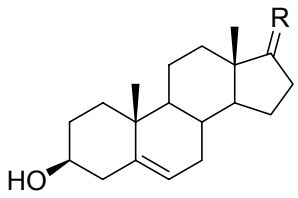


VIII

VIII - santonin

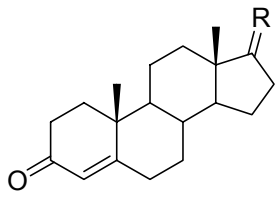
The incubation of some of these terpenes and others (e.g. **V-VIII**) as well as their analogues with the fungi *Beauveria bassiana*, *Rhizopus arrhizus*, *Mucor plumbeus*, *Curvularia lunata* and *Aspergillus niger* has been effected.

This has led to the preparation of a wide range of compounds, many which are products of hydroxylation. Some of these metabolites are novel and a number possess enhanced biological activity[9-14].



IX: R=O
X: R=βCH$_3$CO, αH

IX - DHEA
X - pregnenolone



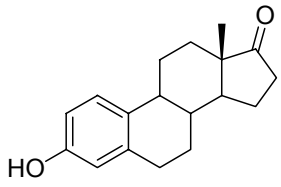
XI: R=βOH, αH
XII: R=βCH$_3$CO, αH

XI - testosterone
XII - progesterone

The fungus *Fusarium oxysporum* var. *cubense* is the causative agent of the dread Panama disease of bananas (*Musa* sp.).

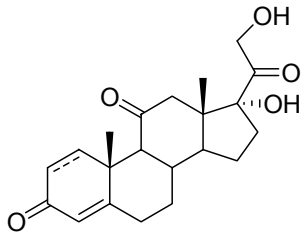
This deuteromycete grows well in liquid culture and has been found to bring about 7α hydroxylation on steroids **IX** and **X**. Substrates **XI-XIII** were functionalised in the C-15α position. **XIV** and **XV** underwent side chain cleavage.

Some redox reactions also accompanied these transformations[15].



XIII

XIII - estrone



XIV: Δ^4
XV: $\Delta^{1,4}$

XIV - cortisone
XV - prednisone

Exophiala jeanselmei var. *lecanii-corni* was encountered as a contaminant of a ginger plant (*Zingiber officinale*). The fungus, which belongs to the Ascomycotina, effected side chain degradation on **X**, **XIV** and **XV**; 1,2- and 1,4 reduction of enones **XI**, **XIV** and **XV**; and redox chemistry on most substrates. Epoxidation, followed by rearrangement, was observed when **IX** was incubated with the fungus[16].

The feasibility of terpene transformation, by these fungi, will be investigated in the future.

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A NEW EFFECTIVE APPROACH TO 6α-METHYLHYDROCORTISONE

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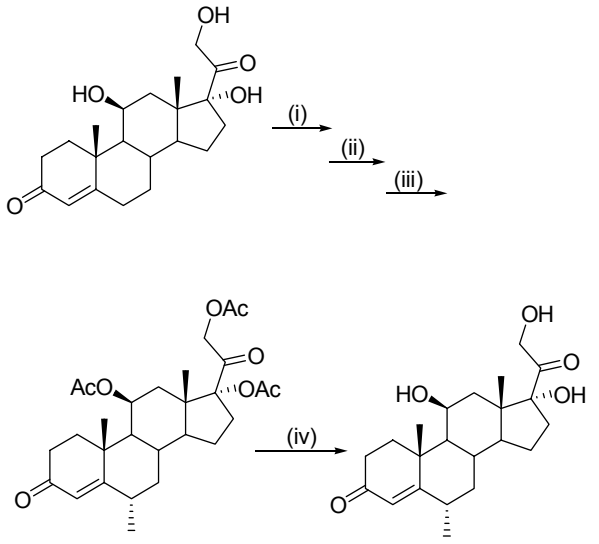
6α-methylprednisolone (medrol, methypred, etc.) is among very important pharmaceuticals with anti-inflammatory, antiallergenic and immunosuppressant effect, which is by 6-7 times as active as its nearest analogue – prednisolone. Moreover 6α-methylprednisolone does not posses mineral corticoid (sodium-arresting) after-effect. A propitious approach to 6α-methylprednisolone is a microbiological 1,2-dehydrogenation of 6α-methylhydrocortisone[1,2]. Although the latter compound is of vital importance, there are very few publications which deal with its chemistry and synthesis[3].

We have reproduced these approaches and discovered that they all are extremely laborious and time-consuming, requiring meticulous protection/deprotection procedures to ensure tolerance to the existing functionality thus leading to essential losses and decrease of net yield. As the synthesis of hydrocortisone from naturally occurring sterols (*via* androst-4-en-3,17-dione and then cortexolone 21-acetate) is a well-known and optimised procedure[2], we decided that an effective approach to 6α-methylhydrocortisone synthesis should proceed through a triester of hydrocortisone.



(i) Ac_2O, pyridine, CH_2Cl_2; (ii) $(MeO)_2CH_2$, $POCl_3$, NaOAc, $CHCl_3$; (iii) Pd/C, cyclohexene, EtOH, (iv) *Corynebacterium mediolanum ABT-AL-301*

We have found that hydrocortisone can be easily transformed to its 11,17,21-triacetate in 98% yield. Introduction of 6-methyl group can be achieved via methylenation[4] or through Vilsmeier formylation[5] with subsequent hydrogenation in 70% total yield. It is known that subsequent hydrolysis of all ester groups is complicated by an easy elimination of hydroxyacetyl group in 17-position. We have shown that more than 97% yield of 6α-methylhydrocortisone can be achieved during hydrolysis of starting triester by means of *Corynebacterium mediolanum ABT-AL-301*.

The data obtained allow us to improve industrial synthesis of 6α-methylprednisolone from sterols in more cost effective way.

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TERPENE FORMATION IN MAIZE AND ITS ECOLOGICAL AND EVOLUTIONARY SIGNIFICANCE

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Despite the remarkable abundance and diversity of terpenoid secondary metabolites in plants, there are still large gaps in our knowledge of their biological functions and evolutionary origin. However, the availability of genetic and genomic resources for certain model plant species provides an exciting array of new tools for exploring the ecological and evolutionary significance of this enormous class of natural products. We have begun to employ genetic and genomic tools to study terpene biosynthesis in corn (*Zea mays)*, focusing on the genes of the terpene synthase family which encode the major group of enzymes controlling the formation of terpenoid secondary metabolites. By carrying out sequence comparisons, functional characterization and gene expression studies along with profiling terpenoid metabolites, we have gained new information about the physiology, ecology and evolution of monoterpenes and sesquiterpenes in this species.

The C_{10} and C_{15} terpenoids of maize are not associated with specialized oil cells, ducts, trichomes or other secretory cavities and are present at low levels throughout the plant. Damage to the plant by lepidopteran larvae, such as the beet army worm (*Spodoptera exigua*) and the European corn borer (*Ostrinia nubilalis*), results in the release of volatiles, including terpenoids, indole, and products of the lipoxygenase pathway

into the headspace of the plant. This volatile blend attracts herbivore enemies like the parasitoid *Cotesia marginiventris*, a braconid wasp (Turlings et al., 1990, 1991). *Cotesia marginiventris* females lay their eggs on *S. exigua* caterpillars and the parasitoid larvae that emerge begin to consume their insect host leading to its eventual death. Since caterpillars parasitized by *C. marginiventris* consume significantly less plant tissue than unparasitized caterpillars (Turlings and Fritzsche, 1999), such tritrophic interactions can be of significant benefit to the plant and are termed indirect defense. In addition, the volatility and reactivity of these substances support a protective function against oxidative damage, analogous to that proposed for isoprene.

The universal occurrence of monoterpenes and sesquiterpenes in higher plants also argues that these substances appeared early in angiosperm evolution. If so, how can one account for the bewildering differences in terpene composition within and among plants? It has previously been established that within plant diversity can often be attributed to the ability of individual terpene synthase enzymes to make multiple products. Indeed, we have found that multi-product terpene synthases appear to be just as prevalent in maize as in classical terpene-accumulating species, such as labiates and conifers. To explain the origin of terpene diversity among grasses, we have employed terpene synthase sequence comparison, mapping experiments and genomic sequence

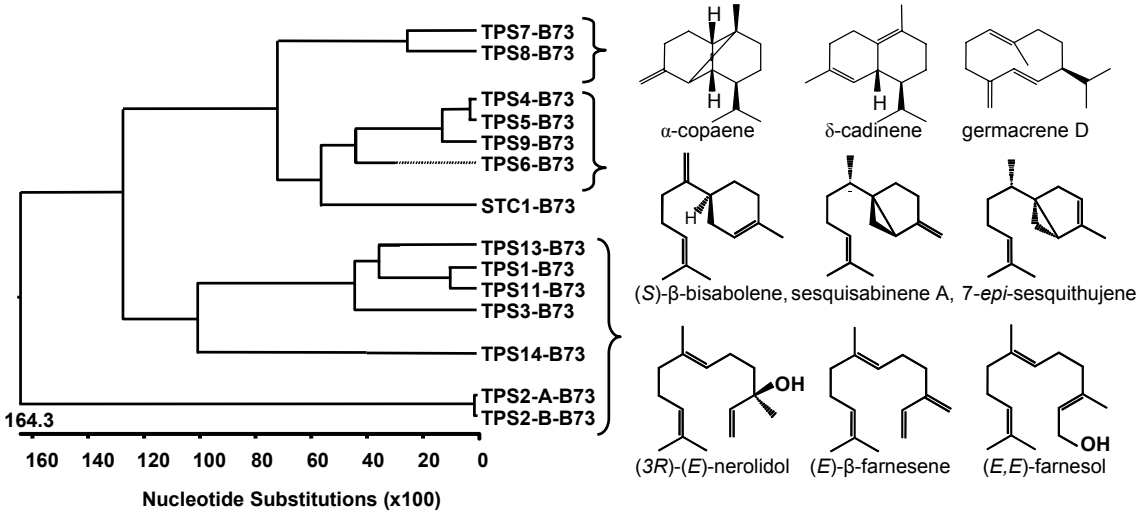

Figure 1: Dendrogram analysis of the maize terpene synthases based on amino acid sequence identity. Examples of the major products of the terpene synthases within each subgroup are given.

information to search for the signatures of past evolutionary processes. Dendrogram analysis of the maize terpene synthases based on amino acid sequence identity revealed a very diverse group of enzymes that forms several subgroups (Figure 1). The enzymes TPS1, TPS2 and TPS11 form acyclic olefins and terpene alcohols from the substrate farnesyl diphosphate. These enzymes share a fairly simple reaction mechanism, but do not necessarily have a high sequence identity which suggests an instance of convergent evolution. The enzymes TPS7 and TPS8 have related amino acid sequences and form mostly bicyclic sesquiterpenes of the cadinane type with a partially overlapping product spectrum. Many of the monocyclic terpenes of maize are formed by the enzyme cluster TPS4, TPS5 and TPS10.

To reconstruct scenarios involving gene duplication and subsequent divergence, we studied the terpene synthases TPS4 and TPS5 in the maize varieties B73 and Delprim. The two enzymes are encoded by separate genes on chromosome 10 and share 96% identity on the amino acid level. Both convert farnesyl diphosphate to a complex blend of approximately 20 sesquiterpenes dominated by sesquithujane-, sesquisabinane-, bergamotane- and bisabolane-type olefins, but the two enzymes favor the formation of distinct stereoisomers resulting in a substantial difference in their product spectra (Figure 2). Site directed mutagenesis revealed that only four amino acid residues in the catalytic center control its stereoselectivity, with the most dramatic change in product profile being observed upon the substitution of an alanine by a glycine. Structural models of the catalytic center suggest that minor changes in the wall of the cavity are causing the stereoselectivity of the enzymes.

To determine why emissions of mature B73 plants are dominated by TPS4 products while those of mature Delprim plants are dominated by TPS5 products, we searched both varieties for alleles of tps4 and tps5. In B73, an active tps4 allele is present, but the protein encoded by the *tps5* allele is catalytically inactive when expressed heterologously.

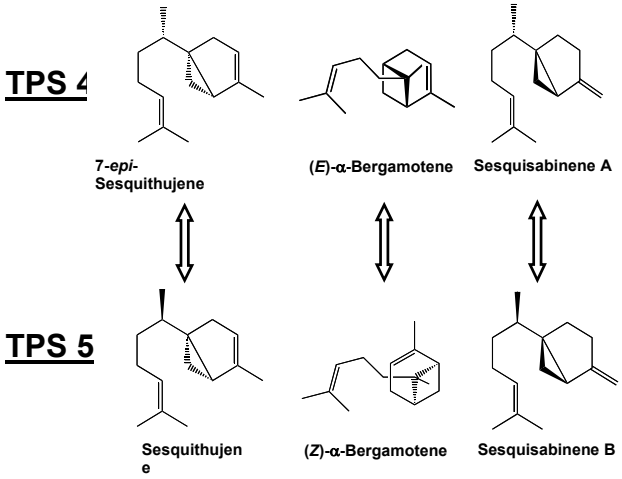

Figure 2: The terpene synthases TPS4 and TPS5 form a very similar group of sesquiterpene olefins with many of the products being stereoisomers of each other.

Site-directed mutagenesis of this inactive allele showed that alteration of two amino acid residues was all that was necessary to render it functional. Conversely, the cultivar Delprim harbors an active *tps5* allele, but its *tps4* alleles are non-functional due to a frameshift mutation. These results suggest that substantial differences in terpene profiles can be controlled by alleles possessing only minor sequence differences.

It is most likely that *tps4* and *tps5* are the result of a recent gene duplication and diversification within the last five million years. Many studies have observed that after gene duplication, one of the two duplicated genes loses its activity due to the functional redundancy of the encoded proteins. This loss of activity might proceed via the gradual accumulation of non-functional alleles within the plant population. It is conceivable that this process is responsible for the surprisingly high number of inactive *tps4* and *tps5* alleles. Since terpene synthases are probably not be essential for plant survival under many growing conditions, the accumulation of mutations might be somewhat higher than in genes of primary metabolism. The higher mutation rate affecting the product specificity of the terpene synthase might be advantageous to the plant to acquire new defenses against large numbers of constantly adapting enemies. The strong expression of the terpene blends in leaves and husks in plants after anthesis suggests a role as toxin or feeding deterrent. Further studies will be necessary to evaluate the contribution of sesquiterpenes to the defense against herbivores or fungal pathogens of maize.

LIGAND RECOGNITION BY PROGESTERONE RECEPTORS FROM FILAMENTOUS FUNGUS *RHIZOPUS NIGRICANS* EXTENDS TO ARYLHYDROCARBONS AND FLAVONOIDS

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A saprophytic fungus *Rhizopus nigricans* (*R. nigricans*) can thrive in several natural and artificial conditions since it contains an effective adaptation system. An important part of its capability to acclimatize to several environmental compounds represents the detoxification system containing cytochrome P450 which transforms different fungitoxins into less toxic products[1]. In this way mammalian hormone progesterone is rendered into harmless 11α-hydroxyprogesterone[2]. Progesterone-hydroxylase is inducible by the substrate progesterone[3] and it seems that progesterone receptors are involved in this progesterone singnaling[4]. In the presented study we characterized progesterone receptors in *R. nigricans* cytosol with respect to steroidal and nonsteroidal ligand specificity. In addition, we examined the effect of selected ligands on hydroxylase induction by progesterone.

In competition studies 40 nM (^{3}H)-progesterone was used. Out from steroids 3,20-keto-pregnanes were the best ligands defined by EC_{50} of 2.2±0.5 x 10^{-7} M. Reduction of C3-oxo and elimination of C17 side-chain significantly decreased the affinity for receptors. Among nonsteroidal arylhydrocarbons, known as environmental pollutants, α-naphthoflavone (EC_{50}=3.2±0.4 x 10^{-8} M), β-naphthoflavone and benzo(a)pyrene competed effectively with progesterone for progesterone receptors, but β-naphthoflavone and benzo(a)pyrene were not

able to displace labeled progesterone to the same level as nonlabeled progesterone. Most likely, these compounds are bound to an allosteric site. Moreover, some natural flavonoids were examined as progesterone receptor ligands. The obtained competition curves did not reach the bottom level of nonlabeled progesterone; their apparent EC_{50} values were as follows: flavone, 1.9±0.8 x 10^{-6} M; kaempferol, 1.7±0.6 x 10^{-6} M; apigenin, 8.1±0.9 x 10^{-6} M; isoflavone genistein, 1.1±0.8 x 10^{-5} M.

Progesterone receptor ligands were used as inducers of progesterone hydroxylase. A clear dose-dependent enzyme induction was obtained with high affinity ligands (progesterone, deoxycorticosterone and testosterone), whereas no induction was observed with low affinity ligand estradiol (Fig. 1). Furthermore, nonsteroidal good progesterone competitors were examined for their ability to interfere with hydroxylase induction by progesterone. α-naphthoflavone inhibited the induction in a dose-dependent manner, whereas the inhibition by benzo(a)pyrene was not dose-dependent (Fig. 2). The antagonistic action of α-naphthoflavone strongly confirms the involvement of progesterone receptors in progesterone signaling resulting in 11α-progesterone hydroxylase induction.

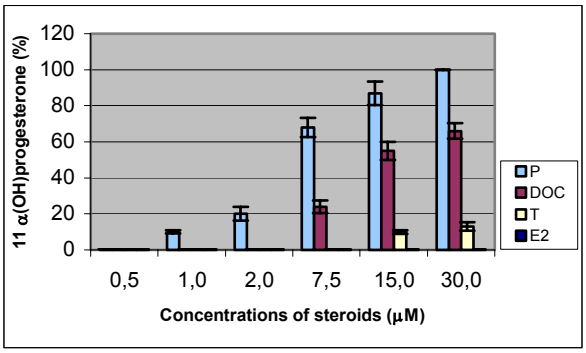

Fig. 1. Dose dependence of progesterone-hydroxylase induction by steroids.

In Fig 1 we can see that after growing for 18 hours *R. nigricans* was incubated for additional 2 hours with steroids of concentrations as indicated. Subsequently, a hydroxylation activity test was performed using progesterone as substrate. Results are presented in percent of conversion of progesterone into 11α-hydroxyprogesterone where the maximal conversion obtained by 30 μM progesterone was defined as 100%. P, progesterone; DOC, deoxycorticosterone; T, testosterone; E2 estradiol

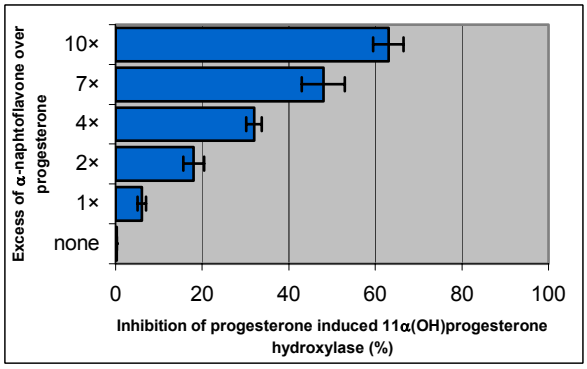

Fig. 2 A. Inhibition of progesterone induction of progesterone hydroxylase with simultaneous addition of indicated excesses of α-naphthoflavone

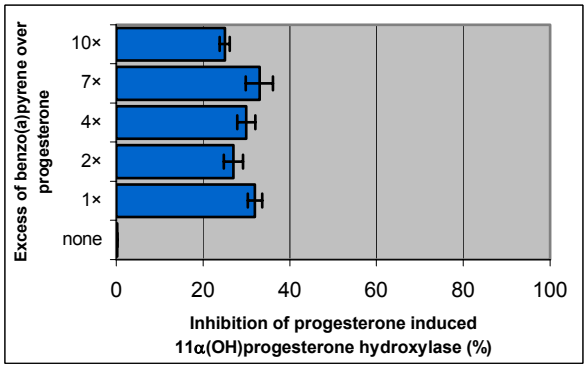

Fig. 2 B. Inhibition of progesterone induction of progesterone hydroxylase with simultaneous addition of indicated excesses of benzo(a)pyrene.

R. nigricans was grown for 18 hours and for additional 2 hours with 15 μM progesterone alone or with simultaneous addition of indicated excesses of (Fig. 2 A) α-naphthoflavone and (Fig. 2 B) benzo(a)pyrene. Hydroxylation activity test was performed using progesterone as substrate and the yield of 11α-hydroxyprogesterone determined. Results are presented in percent of inhibited progesterone-induced hydroxylase activity.

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