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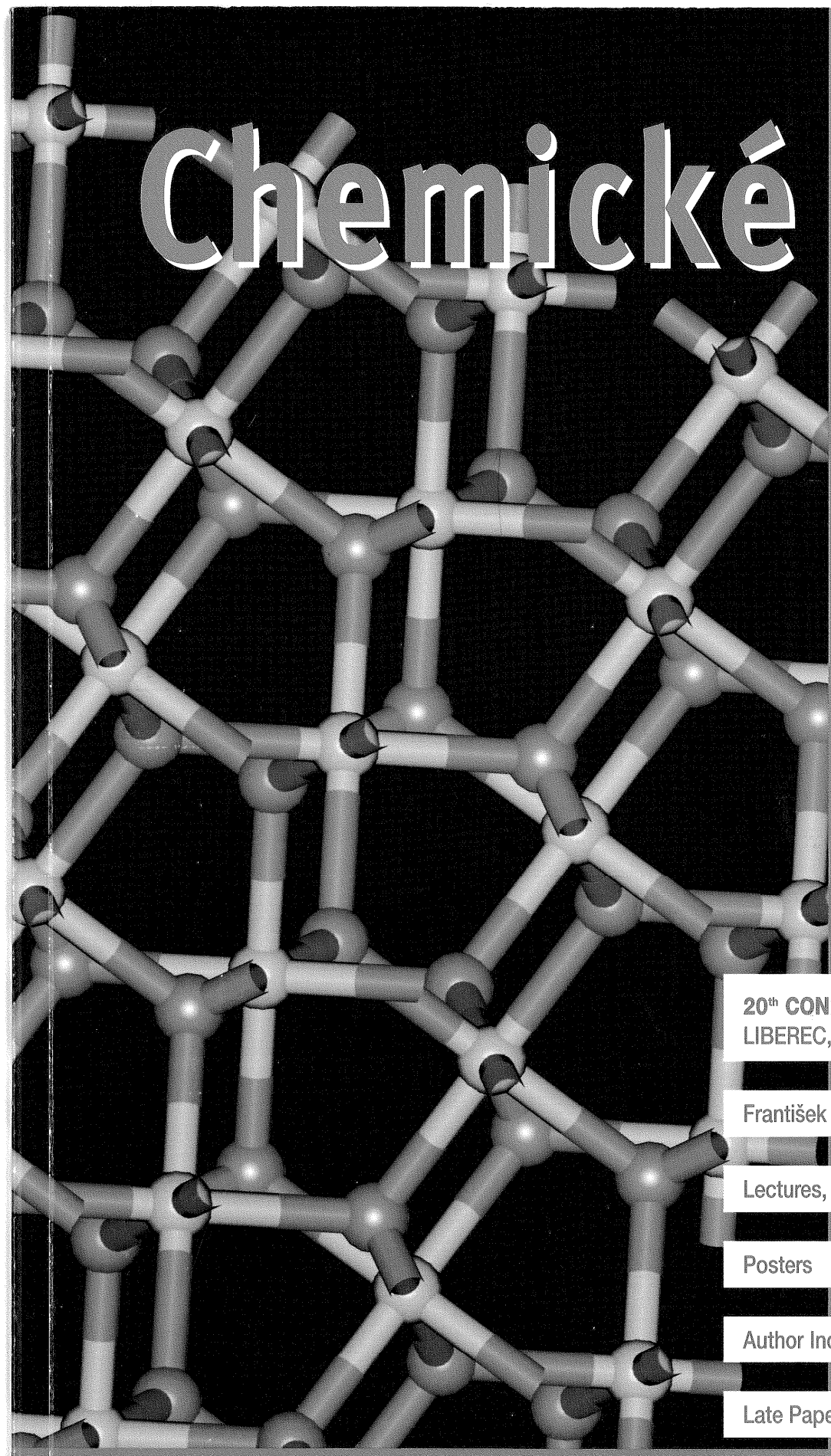

20th CONFERENCE ON ISOPRENOIDS 2003
LIBEREC, CZECH REPUBLIC

Ročník 97
CHLSAC 97 (S) s233 - s327 (2003) ISSN 0009 - 2770 http://chemicke-listy.vscht.cz

Chem. Listy 97, s319-s327 (2003)

A NEW EFFECTIVE APPROACH TO 6α-METHYLHYDROCORTISONE

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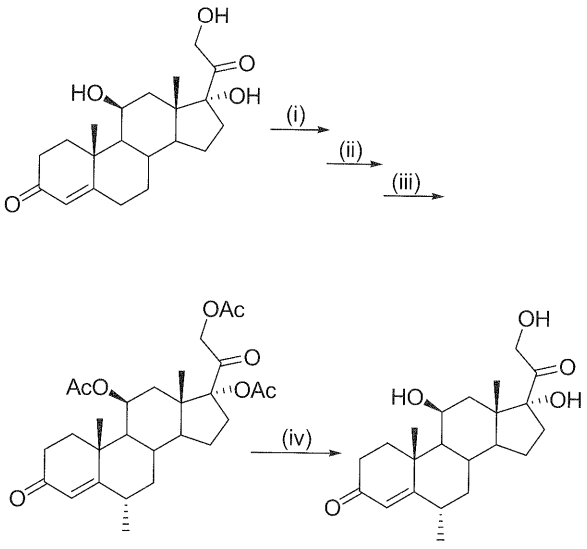
6α-methylprednisolone (medrol, methypred, etc.) is among very important pharmaceuticals with anti-inflammatory, antiallergenic and immunosuppressant effect, which is by 6-7 times as active as its nearest analogue – prednisolone. Moreover 6α-methylprednisolone does not posses mineral corticoid (sodium-arresting) 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

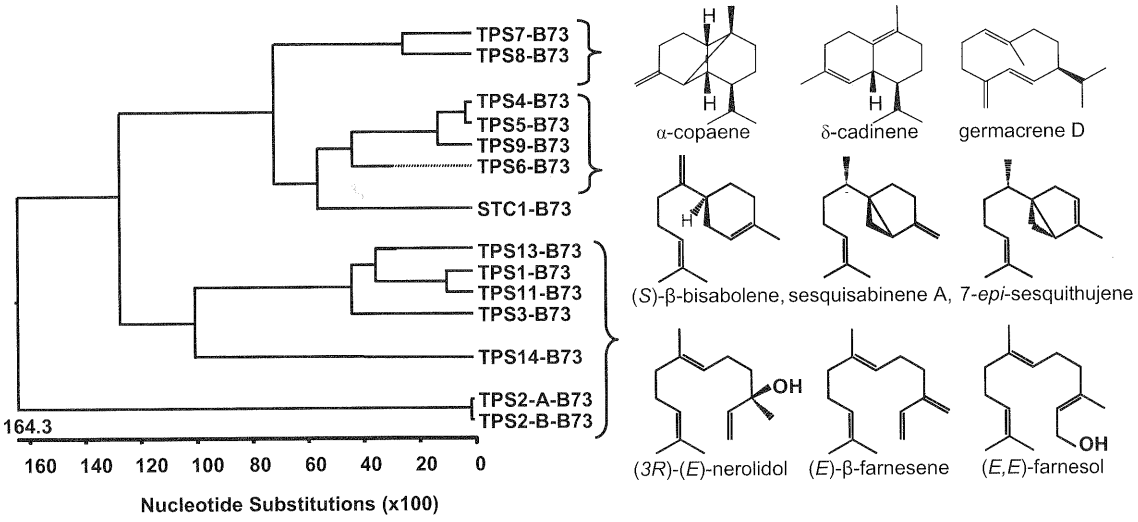

Figure 1: Dendrogram analysis of the maize terpene synthases based on amino acid sequence identity. Examples of the major products of the terpene synthases within each subgroup are given.

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.

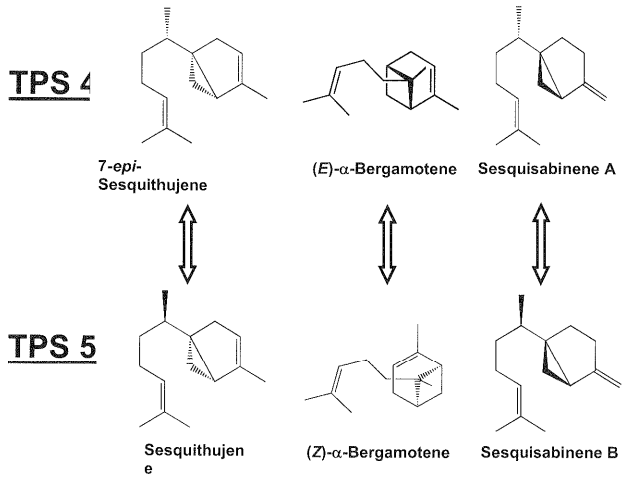

Figure 2: The terpene synthases TPS4 and TPS5 form a very similar group of sesquiterpene olefins with many of the products being stereoisomers of each other.

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