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(*R*)-(+)-PALASONIN, A CANTHARIDIN-RELATED PLANT TOXIN, ALSO OCCURS IN INSECT HEMOLYMPH AND TISSUES

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Abstract—Gas chromatographic and mass spectroscopic analyses of extracts of cantharidin-containing meloid, clerid, and staphylinid beetles revealed the presence of minor to significant amounts of palasonin, previously only known from seeds and fruits of the Indian shrub *Butea frondosa* (Leguminaceae). Unlike (*S*)-(−)-palasonin (>99% ee) from *B. frondosa*, the insects produce palasonin of low ee with the (*R*)-(+)-enantiomer (0–50% ee) prevailing. The ee of palasonin from individual specimens of predatory insects (*Trichodes apiarius*), which acquire their chemical protection from cantharidin-producing insects, may vary considerably. The absolute configuration of (*S*)-(−)-palasonin, previously deduced from indirect chemical and spectroscopic methods, was confirmed by X-ray crystal structure analysis of a cyclic imide derived from (*S*)-(−)-palasonin and (*S*)-(−)-1-(4-nitrophenyl)-ethylamine.

Key Words—Cantharidin, palasonin, *Butea frondosa*, Meloidae, Cleridae, Staphylinidae, canthariphilous insects.

INTRODUCTION

The terpenoid cantharidin represents one of the most famous defensive compounds from insects. Blister beetles (Meloidae) and false blister beetles (Oedemeridae) are

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the only insect species that are able to synthesize this unusual vesicant, insecticide, antifeedant (Carrel and Eisner, 1974; Carrel et al., 1986), and powerful inhibitor of protein phosphatase 2A (PP2A) (Matsuzawa et al., 1987). Since the dynamics of protein phosphorylation and dephosphorylation by protein kinases and protein phosphatases control metabolic activities, cantharidin affects one of the most important regulatory elements of cellular signal transduction (Cohen, 1989). Besides the meloids and oedemerids, other insect orders, collectively called cantharidphilous insects, utilize cantharidin for protection (Dettner, 1997). These insects are not able to produce cantharidin by themselves but are attracted over distances to sources of the toxin and become protected after uptake of the compound from the hemolymph of living or dead meloids, oedemerids, or other cantharidin sources (Frenzel et al., 1992; Dettner, 1997; Hemp and Dettner, 2001). In several species, male-produced cantharidin is transferred during copulation to the female, for incorporation into the eggs (Holz et al., 1994; Eisner et al., 1996).

A structurally related toxin, namely (−)-palasonin, which lacks one of the two angular methyl groups of cantharidin, was isolated in 1967 from seeds of the Indian shrub *Butea frondosa* Koenig ex Roxb. (Leguminaceae), also known as "flame of the forest" due to its brightly orange colored flowers (Raj and Kurup, 1967). Palasonin was found to be effective against ascarids, stomach worms of sheep, oxyurids of mice, and hook worms of dogs (Mehtar and Parasar, 1966). The biochemical mechanism of the antihelminthic action of palasonin is based upon inhibition of glucose uptake affecting the energy generating mechanisms and motor activity of the parasite (Kumar et al., 1995). Like cantharidin, palasonin also inhibits PPA2, albeit with much lower efficiency (Liu et al., 1995).

Although both compounds share the same unique, angularly methylated oxabicyclo skeleton, the toxins have never been reported to cooccur in either plants or insects. However, recent analyses of species from the families Meloidae (Dettner et al., 2001), Cleridae, and Staphylinidae gave the first evidence for simultaneous presence of cantharidin and palasonin in the hemolymph and tissues of individual insects. Since palasonin is a chiral compound, we not only thoroughly documented the occurrence of the two toxins in cantharidin-producing and cantharidin-utilizing insects, but also investigated the absolute configuration and enantiomeric excess (ee) of the compound.

METHODS AND MATERIALS

Collection of Insects

Specimens of Hycleaus lunata Pall. (Meloidae) were caught in 1996 by Prof. Dr. G. Gäde (Rondebosch, South Africa) and transported to Bayreuth by Prof. Dr. E. Zebe (Münster), where they were frozen. Specimens of *Trichodes apiarius* (L.) (Cleridae) were caught from yellow colored composite blossoms at

Saint Jean du Gard (Southern France, Département Gard) on May 30, 1993 by K. Dettner. Rove beetles of *Eusphalerum minutus* (F.) (Staphylinidae) were collected from *Ranunculus* blossoms by K. Dettner near Bayreuth-Seulbitz (Northern Bavaria, Germany) on May 10, 1996.

Extraction of Beetles

Insects were extracted following a standard protocol. Prior to extraction, dry body weight was determined for all samples. Whole specimens, reproductive organs of beetles, eggs, or larvae were treated in small test tubes with 50–300 μl 6N hydrochloric acid (HCl) at 120°C for 4 hr in order to dissolve all body structures and release bound material. Following hydrolysis, equivalent amounts of $CHCl_3$ (50–300 μl) were added and samples were vigorously shaken on a Vortex mixer for 30 sec. Layers were separated by centrifugation at 2000*g* for 3 min. The lower organic phase was filtered, transferred to a vial, and covered with water (10–50 μl). Samples were taken from the lower $CHCl_3$ layer and directly analyzed by GLC-MS.

Isolation of (−)-Palasonin from Seeds of Butea frondosa

Powdered seeds of *Butea frondosa* (140 g) were placed into a Soxhlet apparatus (500 ml) and defatted with low boiling petrol ether (40–60°C, 800 ml) for 24 hr. The seed powder was soaked over night at room temperature in a mixture of *i*-propanol and conc. HCl (500:5 ml). Solids were removed by suction followed by concentration of the solution under reduced pressure and acidification with 2N HCl. Palasonin was thoroughly extracted from the aq. solution with $CHCl_3$ (3×20 ml). After removal of the solvent, the crude brown oil was redissolved in ether (10 ml) and coated onto silica gel (1 g). The solid support was placed into a Soxhlet apparatus (100 ml) and continuously extracted for 16 hr with a mixture of petroleum ether–ether (1:3, v/v). After removal of the solvents, the crude residue (ca. 1.3 g) was recrystallized from low boiling petroleum ether (40–60°C) and yielded 0.47 g of (−)-palasonin. Spectroscopic data and the chiroptical properties (CD and ORD) were in agreement with the literature (Peter et al., 1974).

1-(4-Nitrophenyl)-Ethylimide of (−)-Palasonin

Crystalline (−)-palasonin (0.26 g, 1.42 mmol) isolated from seeds of *Butea frondosa* and (*S*)-(−)-1-(4-nitrophenyl)-ethylamine (0.18 g, 1.08 mmol) were mixed in a small reaction vial (4 ml) and heated to 140°C for 30 min. After cooling, the product was dissolved in CH_2Cl_2 (20 ml) and successively washed with 1N NaOH (15 ml), 1N HCl (15 ml), and water (15 ml). After removal of solvents, the crude product was purified by chromatography on silica gel using a binary

solvent of petroleum ether (40–60°C) and ether (3:5 v/v) for elution. Recrystallization of the condensation product from petroleum ether–ether (3:1 v/v) afforded the imide as colorless needles. Yield: 0.10 g (21%). Mp: 111°C. $[\alpha]_{589}^{26} = 78.8°$ ($c = 0.025$ g/ml, methanol). ^{1}H NMR (acetone-d_6) δ [ppm]: 1.34 (s, 3H); 1.61–1.73 (m, 2H), 1.75–1.83 (m, 1H), 1.79 (d, $J = 7.0$, 3H), 1.95–2.01 (m, 1H), 2.59 (s, 1H), 4.56 (d, $J = 4.9$, 1H), 4.61 (d, $J = 5.5$, 1H), 5.43 (q, $J = 7.1$, 1H), 7.61 (d, $J = 8.85$, 2H), 8.18 (d, $J = 8,85$, 2H). ^{13}C NMR (acetone-d_6) δ [ppm]: 16.1, 17.0, 24.7, 28.9, 49.8, 53.7, 56.7, 81.5, 83.3, 124.2, 128.6, 148.6, 148.5, 177.3, 181.7. IR (KBr) ν [cm^{-1}]: 3050, 1751, 1699, 1516, 1343, 1392, 858. MS (70 eV) *m/z* (%): 330 ($M^{+\bullet}$, 100), 312(20), 300(15), 285(20), 262(60), 244(15), 208(15), 151(60), 113(40), 82(40), 70(60). HR-MS *m/z* [M^+]: calcd. for $C_{17}H_{18}N_2O_5$ 330.121572; found 330.121643.

Crystal Structure Determination

The intensity data for the compound were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo-K$_\alpha$ radiation. Data were corrected for Lorentz and polarization effects, but not for absorption [COLLECT, Data Collection Software; Nonius B.V., Netherlands, 1998 (Otwinowski and Minor, 1997)]. The structure was solved by direct methods (SHELXS; Sheldrick, 1990) and refined by full-matrix least-squares techniques against Fo^2 (SHELXL-97; Sheldrick, 1993). The hydrogen atoms were located by difference Fourier synthesis and refined isotropically. All nonhydrogen atoms were refined anisotropically (Sheldrick, 1993). XP (Siemens Analytical X-ray Instruments, Inc.) was used for structure representations.

Crystal Data for 1-(4-Nitrophenyl)-Ethylimide of (−)-Palasonin

$C_{17}H_{18}N_2O_5$; Mr = 330.33 g/mol, colorless prism, size 0.18 × 0.10 × 0.08 mm^3, monoclinic, space group $P2_1$, $a = 11.3905(8)$, $b = 6.5844(6)$, $c = 12.0322(8)$ Å, $\beta = 117.016(4)°$, $V = 803.9(1)$ Å^3, $T = -90°$C, $Z = 2$, $\rho_{\text{calcd.}} = 1.365$ g/cm^3, μ (Mo-K$_\alpha$) = 1.02 cm^{-1}, $F(000) = 348$, 3134 reflections in $h(-14/14)$, $k(-7/8)$, $l(-15/15)$, measured in the range $3.40° \leq \Theta \leq 27.40°$, completeness $\Theta_{\max} = 99.1\%$, 3134 independent reflections, 2573 reflections with $F_o > 4\sigma(F_o)$, 289 parameters, 1 restraints, $R1_{\text{obs}} = 0.085$, $wR2_{\text{obs}} = 0.112$, $R1_{\text{all}} = 0.115$, $wR2_{\text{all}} = 0.120$, GOOF = 1.149, Flack parameter of 1.7(16), largest difference peak and hole: 0.207/−0.236 e Å^{-3}. Further details of the crystal structure investigation are available on requests from the director of the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK, on quoting the depository CCDC 168993, the names of the authors, and the journal citation.

Detection of Cantharidin and Palasonin and Chiral Analysis

Detection. Five microliters of each beetle extract was injected by using split/splitless injection in a capillary gas chromatograph (GCQ system, Finnigan MAT) equipped with a HT5 fused silica capillary column (bonded phase, SGE; 25 m × 0.32 mm ID, 0.1 μm phase thickness) connected to an ion trap system (GCQ). Electron impact ionization (70 eV) of all samples investigated provided mass spectra with characteristic fragment for palasonin ($M^{+\bullet}$: 182, *m/z* = 114, 82) and cantharidin ($M^{+\bullet}$: 196, *m/z* = 128, 96).

Chiral Analysis. The chiral analysis of palasonin was performed on a TraceMS (Thermoquest, Egelsbach, Germany) equipped with a silica column coated with Lipodex E (Macherey & Nagel, Düren, Germany) (25 m × 0.25 mm, 0.32 μm). The GC injection port was operated at 220°C and the transfer line at 270°C. The oven temperature was programmed from 80°C to 200°C at 5°C/min, with an initial isothermal time of 5 min and a final isothermal time of 10 min. Helium was used as a carrier gas at 3 ml/min and the sample was injected in the splitless mode. Mass spectra were measured in electron impact (EI) mode at 70 eV. Spectra were usually taken in the full scan mode (*m/z* 50–250, one scan per second). In cases of a very low abundance of cantharidin or palasonin, only the two most relevant fragments at *m/z* 82 and *m/z* = 114 were monitored (SIM mode).

RESULTS

Cantharidin and Palasonin in Insects. Blister beetles protect their eggs with cantharidin. The compound is synthesized by the beetles, sometimes only by the males, which transfer it to the females during copulation, for incorporation into the eggs (McCormick and James, 1987). Using both unlabeled cantharidin and stable-isotope-labeled cantharidin combined with mass spectroscopy for analysis, it was demonstrated that such an interindividual cantharidin transfer at copulation and paternal endowment of this toxin into the offspring is a rather common phenomenon among cantharophilous insects (Holz et al., 1994; Eisner et al., 1996). These studies were greatly facilitated by the rather unique mass spectrum of cantharidin, which displays, among others, two highly abundant even numbered fragments at *m/z* = 128 (80%) and *m/z* = 96 (100%), respectively (Figure 1, insert). In continuation of these studies, we recently observed in several specimens of meloid beetles of the genus *Hycleus* minor amounts of another compound that displayed a similar mass spectrum with two major even-numbered fragments that were shifted to lower masses by 14 mass units (Dettner et al., 2001). The compound displayed a quasimolecular ion at $[M+H]^+ = 183$ (cantharidin: $[M+H]^+ = 197$) and two even-numbered fragments at *m/z* = 114 (80%) and *m/z* = 82 (100%). The identity of the fragments, established by high-resolution and linked-scan techniques,

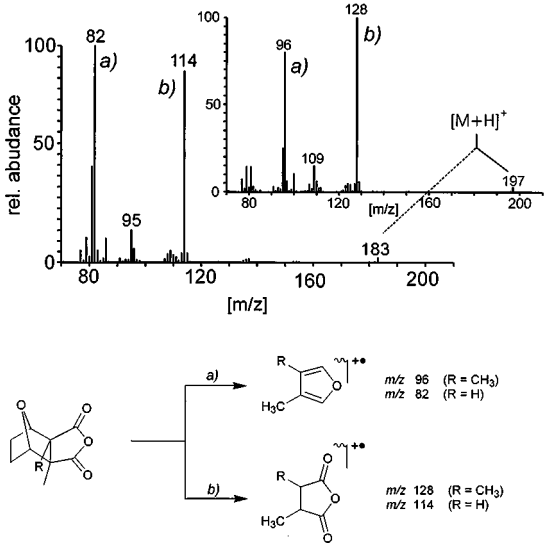

FIG. 1. Mass spectra of cantharidin (insert) and palasonin (main spectrum) from insect extracts. Both compounds are cleaved into the same type of fragments. Methyl-substituted furans (path *a*) and methyl-substituted succinic acid anhydride (path *b*) represent the two most prominent fragments of cantharidin or palasonin.

is outlined in Figure 1 and confirms previous work (McCormick et al., 1986; McCormick and James, 1987).

Due to these characteristic features, the compound was readily identified as palasonin, previously only known to occur in seeds and fruits of the Indian shrub *Butea frondosa*. Within *Hycleus* specimens, there were found 22–321 ng palasonin/mg dry weight (Dettner et al., 2001). Direct comparison of the minor component with authentic palasonin established its identity.

Chiral Analysis of Palasonin. Unlike cantharidin, palasonin is a chiral compound. The lack of the second methyl group introduces dissymmetry and, hence, it is important to know which, and to which extent, individual enantiomers are present in the insects. However, owing to the very low concentration of palasonin in the extracts, only highly sensitive gas chromatographic methods using chiral stationary phases promised to be successful. Optimum results were obtained on an enantioselective capillary column coated with octakis-(3-*O*-butanoyl-2,6-di-*O*-pentyl-γ-cyclodextrin (Lipodex E) that separated racemic palasonin into the two enantiomers with high efficiency ($\alpha = 1.13$).

Figure 2 shows the gas chromatographic separation of palasonin enantiomers from an extract of *Hycleus lunata* Pall a cantharidin-producing meloid. (−)-Palasonin eluted first and the (+) enantiomer second, as established by comparative

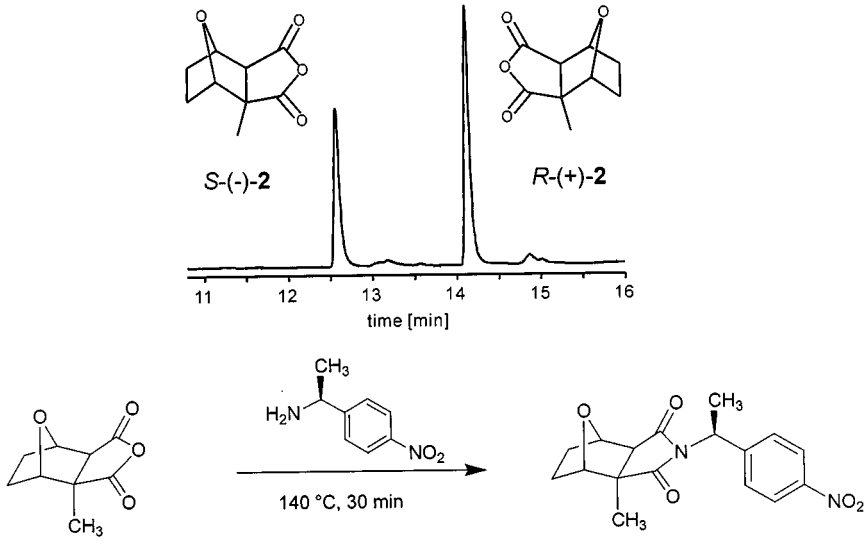

FIG. 2. Gas chromatographic separation of palasonin enantiomers. Palasonin of low ee from an extract of a *Hycleus* sp. is separated on a fused silica column coated with a modified cyclodextrin (Lipodex E). Separation factor: $\alpha = 1.13$.

injection with (−)-palasonin from seeds of *Butea frondosa*. The presence of both enantiomers in the extracts of the meloid beetle *Hycleus lunata* was not unique but proved to be representative for all other hitherto examined palasonin-containing insects. Integration of the peak areas demonstrated that all insect species (Table 1) produced both enantiomers of palasonin with only low to moderate preference for the (+)-enantiomer.

Particularly interesting is the result of the analysis of palasonin from two individuals of the clerid beetle genus *Trichodes apiarius* (L.). One specimen contained almost racemic material, while the other had accumulated (*R*)-(+)-palasonin of 32% ee. Remarkably, this also represents the first record of cantharidin within the beetle family Cleridae. In contrast to palasonin from insects, crude (−)-palasonin obtained from different collections of seeds of *Butea frondosa* proved always to be optically pure (>99% ee).

Absolute Configuration of (+)- and (−)-Palasonin. The absolute configuration of (*S*)-(−)-palasonin from seeds of *Butea frondosa* was originally deduced from its CD spectra and by indirect chemical methods (Peter et al., 1974). The configurational assignment was based on conversion of (−)-palasonin into a secondary alcohol followed by reaction of this intermediate with the anhydride of α-phenylbutyric acid (Horeau, 1961). This empirical method is based on the reaction of secondary alcohols with the above anhydride to give (+)- or

TABLE 1. SPECIMEN ANALYSIS[a]

Toxin origin	Plant or insect species	Cantharidin–palasonin ratio	Palasonin (*S*)-(−) (%)	Palasonin (*R*)-(+) (%)	Palasonin ee (%)
	Leguminaceae				
	Butea frondosa (seeds)	0:100	>99	<1	>98
Cantharidin	Meloidae				
producers	*Hycleus lunata* spec 1	22.4:1	40	60	20
	Hycleus lunata spec 2	205:1	25	75	50
⬇					
Cantharidin	Cleridae				
users	*Trichodes apiarius* spec 1	9.6:1	50	50	0
	Trichodes apiarius spec 1	>99:1	34	66	32
	Staphylinidae				
	Eusphalerum minutum	2.9:1	41	59	18

[a] In *Hycleus lunata* and *Trichodes apiarius* specimens, one individual per sample was analyzed; in *Eusphalerum minutum* four individuals per sample were analyzed.

(−)-α-phenylbutyric acid, depending on the relative size of the substituents flanking the secondary alcohols. Although this indirect method generally proved to be reliable, a direct approach via a crystal structure analysis of a palasonin containing a structural element of known absolute configuration is preferred owing to the unequivocal identification of additional asymmetric centers in the molecule. Palasonin readily reacts with primary amines to give crystalline cyclic imides. A naturally occurring imide of palasonin and (*S*)-(−)-phenylalanin was isolated from seeds of *Butea frondosa* (Barua et al., 1970; Guha et al., 1990). To obtain crystals suitable for structure analysis, we reacted (−)-palasonin (>99% ee; isolated from seeds of *Butea frondosa*) with either (*R*)-(+)- or (*S*)-(−)-1-phenyl-ethylamine. Although the expected imide was formed in good yield, no suitable crystals could be obtained after chromatographic purification. Better results were obtained by reacting (−)-palasonin with 1-(4-nitrophenyl)-ethylamine (Baumgarten and Peterson, 1960) (Figure 3). The additional nitro group in the *p*-position should enhance the

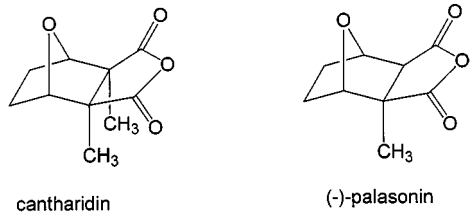

FIG. 3. Reaction of (−)-palasonin from *Butea frondosa* with (*S*)-(−)-1-(4-nitrophenyl)-ethylamine.

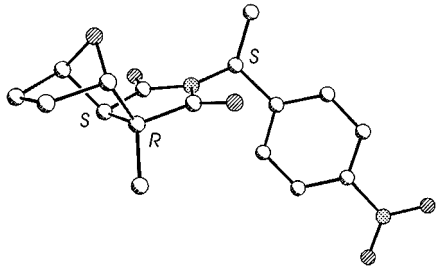

FIG. 4. Molecular structure of the cyclic imide derived from (*S*)-(−)-1-(4-nitrophenyl)-ethylamine and (−)-palasonin as determined by X-ray crystal structure analysis.

tendency of the product to crystallize due to the strongly enhanced intermolecular interactions.

As expected, (−)-palasonin readily reacted with 1-(4-nitrophenyl)-ethylamine to give a cyclic imide that spontaneously crystallized upon removal of the solvent. Recrystallization from a binary mixture of hexane/ether (3:1 v/v) afforded the pure imide. The compound crystallized as monoclinic colorless prisms.

The three-dimensional architecture of the imide is shown in Figure 4. Since the absolute configuration of the asymmetric center next to the aromatic nucleus is known due to the use of (*S*)-(−)-1-(4-nitrophenyl)-ethylamine, the absolute configuration of (−)-palasonin follows as depicted in Figure 4 and unequivocally confirms previous indirect assignments.

DISCUSSION

Cantharidin is an exceedingly powerful toxin that is used by many insects but is only produced by members of two insect families, the meloids and the oedemerids. All canthariphilous and other cantharidin-containing species acquire the toxin from dead or living cantharidin-producing or other cantharidin-containing insects. Specimens of the pollen-feeding staphylinid beetle *Eusphalerum minutum* were regularly found in cantharidin traps, which indicates that they are canthariphilous (Dettner, 1997). Since females of the canthariphilous ceratopogonid *Attrichopogon lucorum* were observed attacking living *E. minutum* by piercing membranes and sclerites of these rove beetles (Klinker, 1983), the presence of palasonin in predatory canthariphilous ceratopogonid flies was expected. Concerning clerid beetles, the search for cantharidin in the genus *Trichodes* hitherto was negative (Juanjie et al., 1995). Therefore, the presence of both anhydrides within certain species of this widespread and abundant beetle family is remarkable. Although it was reported that members of Cleridae are canthariphilous (Bologna and Havelka, 1984; Hemp et al., 1999), specimens of *Trichodes apiarius* were never

observed within cantharidin traps, but occur in various yellow-colored composite blossoms (Dettner, unpublished observations), where they probably feed on cantharidin producing oedemerid and meloid beetles.

Recent analyses of extracts from meloid beetles clearly demonstrated for the first time that palasonin does occur in insects, sometimes even in substantial amounts (Dettner et al., 2001). In general, palasonin was found as a minor or trace compound along with cantharidin as the major toxin (Table 1). As shown by chiral analysis, the insect-derived palasonin is of moderate to very low enantiomeric purity, while the (*S*)-(−)-palasonin from *Butea frondosa* is an optically pure compound. Owing to this major and significant difference between the insect-derived and the plant-produced product, an uptake of palasonin from a hitherto unknown plant source in the environment of meloids and oedemerids appears to be highly unlikely. Hence, it follows that at least the cantharidin-producing meloid beetles must be able to synthesize palasonin *de novo* or by degradation of cantharidin (see below). Moreover, since the various cantharidin-producing specimens of *Hycleus lunata* Pall. generate palasonin with a different ee (Table 1), acquisition of this compound from randomly selected sources by canthariphilous and cantharidin-tolerant predatory insects will inevitably lead to an accumulation of palasonin with varying ee in different individuals of the same species. This phenomenon was observed with two specimens of the canthariphilous genus *Trichodes apiarius* (L.) (Table 1).

Although the mechanistic details of the biosynthesis of cantharidin and/or palasonin are not known, two alternative routes can be envisaged for the origin of palasonin. In the insects, the compound could originate directly from cantharidin (Figure 5, route A) or derive from cyclization of a previously demethylated "12-*nor*-farnesol" (Figure 5, route B) along the pathway analogous to that of cantharidin.

Thus, an oxidative attack onto one of the two angular methyl groups of cantharidin ($-CH_3 \rightarrow -CH_2OH \rightarrow -CHO \rightarrow -COOH$) could finally generate an angular carboxylic acid moiety that, in turn, could readily decarboxylate to palasonin. Owing to the high symmetry of the molecule, a selective functionalization of a distinct methyl group might be difficult and could account for the low ee of palasonin in insects (Figure 5, route A). Alternatively, the oxidative demethylation may occur earlier, on the level of farnesol, the sesquiterpene precursor of cantharidin (Carell et al., 1986) (Figure 5, route B). After oxidative transformation of the two terminal methyl groups into a dicarboxylic acid, one carbonyl group could be lost by decarboxylation. The resulting *nor*- intermediate might be further processed to palasonin, analogous to the pathway to cantharidin. This route might also be operative in seeds or fruits of *Butea frondosa*, since no traces of cantharidin were found along with the main constituent (*S*)-(−)-palasonin. The almost exclusive production of the (*S*)-enantiomer by the plant tissue is also not congruent with the very low ee and the absolute configuration of insect-derived

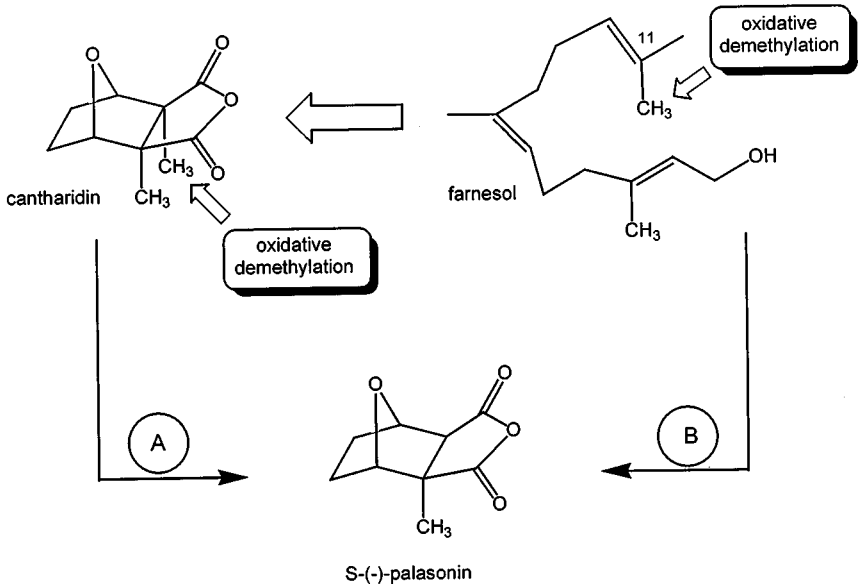

FIG. 5. Alternative biosynthetic routes to palasonin. According to sequence A palasonin is produced by oxidative degradation (demethylation) of cantharidin. Sequence B assumes an early oxidative "demethylation" of farnesol followed by cyclization of the resulting intermediate to palasonin analogous to the biosynthesis of cantharidin (McCormick et al., 1986).

(*R*)-(+)-palasonin and may additionally support an alternative pathway. Although preliminary feeding experiments with larvae of *Oedemera femorata* and synthetic deuterium-labeled 12-*nor*-farnesol precursors demonstrated a successful conversion into labeled palasonin, the amount of the metabolite was too low to assess its configuration and ee (Duetemeyer, 1997; Fietz, 2001). A final decision in favor of a route via late oxidative demethylation (route A) or via demethylation of an early precursor of cantharidin (route B) is, as yet, not possible and awaits further experimental results. Nevertheless, the rather widespread occurrence of palasonin in cantharidin-producing and cantharidin-utilizing insects is unexpected and surprising. Whether or not the compound has a specific ecological function remains to be established and requires further analyses with special emphasis on tissue specific distribution in the insects, its flux between male and female individuals, and, especially, its transfer into eggs for protection of the next generation.

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