Biosynthesis of 1-alkenes in the defensive secretions of *Tribolium confusum* (Tenebrionidae); stereochemical implications

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Received 26 September 1989; accepted 15 January 1990

Summary. The terminally unsaturated hydrocarbons of the defensive secretion of *Tribolium confusum* are biosynthesized from fatty acids by oxidative decarboxylation. The process involves an enantiospecific cleavage of the C-H bond of the *pro*-(S) C(3)-H atom and simultaneous decarboxylation of the acid into an 1-alkene and carbon dioxide via an *anti*-periplanar transition state geometry (*anti*-elimination). The stereochemistry of this biotransformation is identical in all respects with the same reaction in higher plants. The mechanism seems to be of general importance for the biosynthesis of many vinylic substructures of natural products from oxygen-containing precursors.
Key words. Coleoptera: Tenebrionidae; *Tribolium confusum;* defensive secretion; biosynthesis of 1-alkenes; oxidative decarboxylation; *anti*-elimination.

Unsaturated hydrocarbons are synthesized and used by a large number of insects. Their function in the often complex interactions among insects and between insects and their environment is manifold. They may act as alarmones[1], pheromones[2] and repellents[3], or, in combination with defensive secretions as e.g. surfactants, penetrators, evaporatory retardants[4] or even as narcotics for chemoreceptors[5]. As surfactants and biosolvents they improve the effectiveness of the actual chemical weapons like e.g. quinones or iridodials and facilitate their penetration into cuticular structures[4]. Of the identified hydrocarbons ca 25% are terminally unsaturated compounds[6], many of which obviously derive from the pool of fatty acids by oxidative decarboxylation (mostly odd numbered, unbranched 1-alkenes). However, in marked contrast to the well established biological significance of 1-alkenes, almost nothing is known about the mechanism and stereochemistry of their production from fatty acids in insects. Recently, we have shown that 1-alkenes in plants are synthesized from fatty acids by enantiospecific removal of the *pro*-(*S*) C(3)-H atom and fragmentation via an *anti*-periplanar transition state geometry into 1-alkenes and carbon dioxide[7,8]. These results, and the lack of information about insect metabolism that exists at present, prompted us to investigate the biosynthesis of terminally unsaturated hydrocarbons in defensive secretions of insects in more detail, using *Tribolium confusum*, as an example.

The flour beetle *Tribolium confusum*, an easy-to-handle and ubiquitous storage pest, produces a mixture of toluquinone and 2-ethylquinone, together with the corresponding hydroquinones, and also pentadec-1-ene, pentadeca-1,6-diene and smaller amounts of hexadec-1-ene, hexadeca-1,6-diene, hexadecatriene, heptadec-1-ene, heptadeca-1,8-diene and heptadecatriene (cf. fig. 1) in its abdominal glands[9]. This broad spectrum of vinylic hydrocarbons suggested to us that probably 12-phenyl-[^{2}H]dodecanoic acids, which were previously used to unravel the biosynthesis of vinylic C_{17}-hydrocarbons from

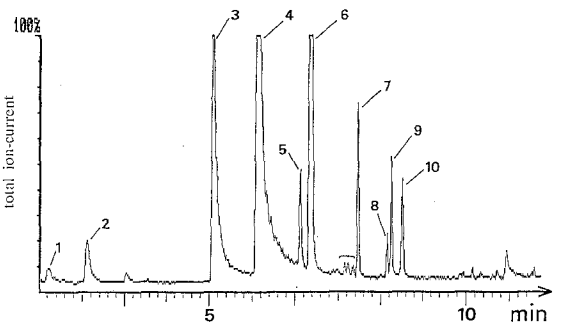


Fig. 1. Gas chromatographic separation of the volatile constituents of the abdominal defensive gland of *Tribolium confusum*. A single reservoir with gland tissue was excised from the abdomen and evaporated at 250 °C after solid injection. Separation was achieved on a 10 m × 0.25 mm fused silica capillary coated with OV 1 under programmed conditions (100 °C for 2 min and then at 15 °C/min to 280 °C). (1) = toluquinone, (2) = 2-ethylbenzoquinone, (3) = 2-methylhydroquinone, (4) = 2-ethylhydroquinone, (5) = pentadeca-1,6-diene, (6) = pentadec-1-ene, (7) = hexadec-1-ene, (8) heptadecatriene, (9) = heptadeca-1,8-diene, (10) = heptadec-1-ene. Compounds marked with a bracket were tentatively identified with reference substances as hexadecadiene and hexadecatriene, respectively. Positions of double bonds are taken from Suzuki[9].

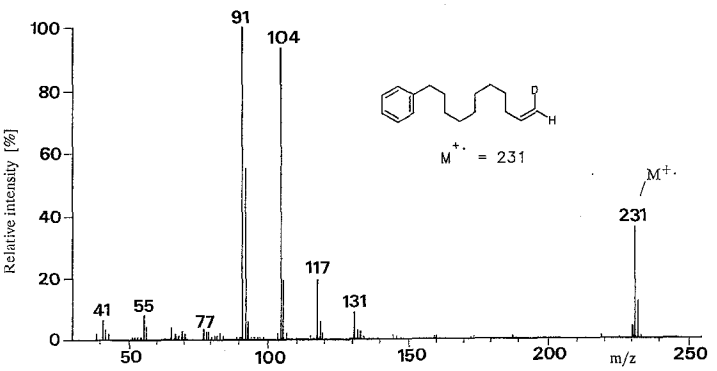


Figure 2. Representative mass spectrum of e.g. 11-phenyl[1-^{2}H]undec-1-ene resulting from administration of (2*S*,3*S*)-11-phenyl[2,3-$^2H_2$]-dodecanoic acid (> 97% e.e. each chiral center) to *T. confusum*. Despite the molecular ion the mass spectra of all other 11-phenyl[^{2}H]undec-1-enes are identical. Metabolites bearing a single deuterium label display an $M^{+\cdot}$ at 231 Da.

C_{18}-precursor acids in higher plants[7,8], might also be useful metabolic probes in insects. The resulting 11-phenyl[-^{2}H]undec-1-enes possess a unique fragmentation pattern (cf. fig. 2) which substantially facilitates their identification among a large number of naturally occurring 1-alkenes. We report here that *Tribolium confusum* is able to convert specifically labelled 12-phenyl[^{2}H]dodecanoic acids to the corresponding 11-phenyl[^{2}H]undec-1-enes, and that the stereochemistry of this oxidative decarboxylation is identical in all respects with that observed in plant metabolism.

Materials and methods

A mixed population of both sexes of *Tribolium confusum* was obtained from the Senckenberg Museum, Frankfurt, FRG. The insects were reared on oat flakes in 0.5-l preserving jars covered with gaze. The containers were kept under a 12-h light regime (1400 lx, daylight fluorescent tubes) at 22–24 °C until the insects were required.

Precursor acids and references. The synthesis of 12-phenyl[2,2-$^2H_2$]dodecanoic acid and 12-phenyl[4,4-$^2H_2$]dodecanoic acid was described earlier[7]. The enantiospecifically deuterated (2*R*,3*R*)-and (2*S*,3*S*)-12-phenyl[2,-3-$^2H_2$]dodecanoic acids ($\geq$ 97% e.e. at each chiral center), (2*R*)-12-phenyl-[2-^{2}H]dodecanoic acid ($\geq$ 97% e.e), 12-phenyl[2,3-$^2H_2$]dodec-2-enoic acid and the reference hydrocarbons (*Z*)- or (*E*)-11-phenyl[1-^{2}H]-undec-1-ene were prepared according to Görgen et al.[10].

Administration of precursor acids. To a solution of 10 mg of each individual ^{2}H-labelled 12-phenyldodecanoic acid in 1 ml Et_2O was added 1 g of oat flakes. The heterogeneous suspension was rotoevaporated in vacuo until the solvent had been quantitatively removed. For feeding experiments ca 30–50 specimens of *T. confusum* were reared for 4 weeks on this precursor-impregnated nutrient under the conditions described above. After this time, the quantity of the artificial metabolites produced was high enough for isolation by chromatography, and subsequent analysis by combined gas chromatography and mass spectrometry (GC/MS). For the infrared spectroscopic analysis ca 1000 specimens were reared for four weeks on 20 g oat flakes impregnated with 0.40 g of (*R*)-12-phenyl[2-^{2}H]dodecanoic acid ($\geq$ 97% e.e.).

Isolation of the artificial metabolites. To minimize a premature secretion of the defensive compounds, the incubated insects (ca 30) were at first slowly cooled to 4 °C and then killed by further cooling to −20 °C. After grinding with 20 ml CH_2Cl_2, the suspension was stirred for 5 h, filtered and concentrated in vacuo. The greasy residue was redissolved in 0.5 ml pentane and chromatographed over a small column of silica gel in a Pasteur pipette (6 cm × 5 mm). The compounds eluted with pentane and were collected in 1.0-ml fractions. Fractions containing the metabolites were combined and concentrated in vacuo to about 60 µl prior to analysis by gas chromatography and mass spectrometry (GC/MS). Purification of the metabolites from the large scale incubation experiments was achieved by chromatography on silica gel (20 × 1 cm) using pentane for elution.

Localization of the artificial metabolites in T. confusum. To monitor the distribution of 11-phenylundecenes within the insects, gland tissue and reservoirs were excised from frozen beetles under the microscope on an ice cooled surface, and deposited into a tiny groove in a cooled wire plunger which is movable within the needle (Mini Injector, Precision sampling Corporation). The material was directly transferred into the injection port of the gas chromatograph, and evaporation at 250 °C was followed by separation on a 10 m × 0.25 mm OV 1 fused silica column and mass spectroscopic detection. Control injections were made using segments of the gut, ovaries or hemolymph collected by the same method.

Analytical methods. Mass spectroscopy (GLC/MS): Routine analyses were performed with a Finnigan ITD 800, combined with a Carlo Erba gas chromatograph, model Vega, equipped with an OV 1 fused silica capillary (10 m × 0.25 mm) for separation of compounds. Elution was programmed routinely from 100 °C (2-min isotherm) to 280 °C at 20 °C/min. Helium at 30 cm/s served as carrier gas. The transfer line was kept at 270 °C, and the scan range was preset at 35-249 Da/s. Isotope analyses were run on a Finnigan MAT 90. Separation of compounds was achieved on a 25 m × 0.32 mm fused silica column coated with DB 5 under programmed conditions from 80 °C (2-min isotherm) to 280 °C at 10 °C/min. The 11-phenyl-undec-1-enes exhibit a characteristic fragmentation pattern, among which the pair of fragments at m/z = 91(100%) and m/z = 104(96%) is most significant (cf. fig. 2). Spectra of the metabolites from samples were compared with spectra of the synthetic references. Owing to the relatively high intensity of the molecular ion ($M^+\cdot$ = 35%; sector field instrument, Finnigan, MAT 90), the degree of deuterium labelling followed directly from the $M^+\cdot$. Gas chromatography (GLC) was carried out using a Carlo Erba gas chromatograph, HRGC 5300 series, equipped with fused-silica capillaries, SE 30 (10 m × 0.31 mm); H_2 at 30 cm/s served as carrier gas. Elution of compounds was achieved under programmed conditions (see above and fig. 1).

Results and discussion

Volatiles from the defensive gland of Tribolium confusum. The hydrocarbon pattern of the paired abdominal sternal glands of *T. confusum* was determined by gas chromatography and mass spectrometry using solid injection of a freshly excised reservoir with gland tissue. As shown in figure 1, the most abundant hydrocarbon is pentadec-1-ene, followed by hexadec-1-ene and heptadec-1-ene, respectively. Some higher unsaturated compounds like

heptadeca-1,8-diene, heptadecatriene, hexadecadiene, hexadecatriene and pentadeca-1,6-diene are also present, but on a lower or even trace level.

Despite the presence of hexadecadiene and hexadecatriene this result is in agreement with solvent extracts from 24,000 insects as reported by Suzuki[9].

Feeding experiments with specifically labelled 12-phenyl(^2H)dodecanoic acids. When specimens of *T. confusum* were reared for four weeks on oat flakes impregnated with either 12-phenyl[2,2-$^2H_2$]dodecanoic acid or 12-phenyl-[4,4-$^2H_2$]dodecanoic acid, GLC/MS analysis of the corresponding extracts immediately showed that the externally added precursors were incorporated and metabolized to give the terminally unsaturated 11-phenyl-undec-1-enes, as had previously been shown for plants[7,8]. GLC retention times and mass spectra of the products were in full agreement with synthetic reference samples of 11-phenyl[1,1-$^2H_2$]undec-1-ene or 11-phenyl[3,3-$^2H_2$]undec-1-ene, respectively. The identical molecular ions of the olefinic metabolites at 232 Da showed that both acids must have been decarboxylated to the 1-alkenes with one carbon less, with quantitative retention of deuterium from the original methylene groups at C(2) or C(4) of the precursor acids (eqns 1 and 2).

$$R-CH_2-CH_2-CD_2-COO^- \xrightarrow{-[H]} R-CH_2-CH=CD_2+CO_2 \quad (1)$$

$$R-CD_2-CH_2-CH_2-COO^- \xrightarrow{-[H]} R-CD_2-CH=CH_2+CO_2 \quad (2)$$

Steric course at C(3). To clarifiy the number of the hydrogens from the methylene group at C(3) involved, as well as the site specificity of the enzyme(s) in *T. confusum*, the two enantiospecifically deuterated (2*R*,3*R*)- and (2*S*,3*S*)-12-phenyl[2,3-$^2H_2$]dodecanoic acids ($\geq 97\%$ e.e., each) were administered, and the products were analyzed as before. The metabolite resulting from (2*S*,3*S*)-12-phenyl[2,3-$^2H_2$]dodecanoic acid displayed molecular ions at 232 Da (loss of ^1H) and 231 Da (loss of ^2H) in a ratio of ca. 1:5 (corrected for the natural abundance of ^{13}C), and indicates a preferential cleavage of the C(3)-D bond as shown in figure 2. In contrast, the enantioisomeric (2*R*,3*R*)-acid is converted with complete retention of deuterium to 11-phenyl-[1,2-$^2H_2$]undec-1-ene ($M^{+\cdot} = 232$ Da). The latter result was confirmed in several runs, but the isotope ratio ($M^{+\cdot}$:232/231) of the metabolite from incubations with the (2*S*,3*S*)-acid showed some variation and is indicative of an intramolecular isotope effect.

As a result of the above feeding experiments it is now evident that only the *pro*-(*S*) C(3)-H atom and the carboxyl group of a fatty acid are involved in the enzymatic reaction leading to terminally unsaturated hydrocarbons. As a matter of fact, the biosynthesis of 1-alkenes in higher plants (e.g. *Carthamus tinctorius,* Asteraceae) involves the same events and shows identical specificity for the *pro*-(*S*) C(3)-H atom of the precursor acid.

Overall stereochemical course; transition state geometry After evaluation of the site specificity of the 1-alkene forming enzymes, the transition state geometry of the fatty acid degradation, i.e. *syn*- or *anti*-elimination, was examined. If the precursor acid is enantiospecifically labelled at C(2), the configuration of the resulting 11-phenyl[1-^2H]undec-1-ene can be used to monitor the overall stereochemical course of the fragmentation, as outlined in figure 3. Since in this experiment there is no deuterium label at C(3) of the precursor acid, the transition state geometry should be accessible without complications due to the intramolecular isotope effect.

Thus, in the case of an *anti*-elimination (*anti*-periplanar orientation of the *pro*-(*S*) C(3)-H atom and the carboxyl group), feeding of (2*R*)-12-phenyl[2-^2H]dodecanoic acid to *T. confusum* should result in a configurationally homogeneous (*E*)-11-phenyl[1-^2H]undec-1-ene. In the case of a *syn*-elimination the pure Z-isomer has to be expected. Since the stereoisomeric 11-phenyl[1-^2H]undec-1-enes exhibit specific infrared bands in the region of 1000-600 cm^{-1}, their configuration can be readily assessed by combined gas chromatography and infrared spectroscopy. The (*E*)-isomer shows a vinyl twist band at 980 cm^{-1}, while the (*Z*)-isomer displays a typical vinyl wagging band at 802 cm^{-1}. The absorbances at 1030, 740 and 690 cm^{-1} (fig. 4) are common to both isomers. However, due to the lower sensitivity of the method and at the same time very low intensity of the characteristic absorbances, about 1000 specimens of *T. confusum* were required to

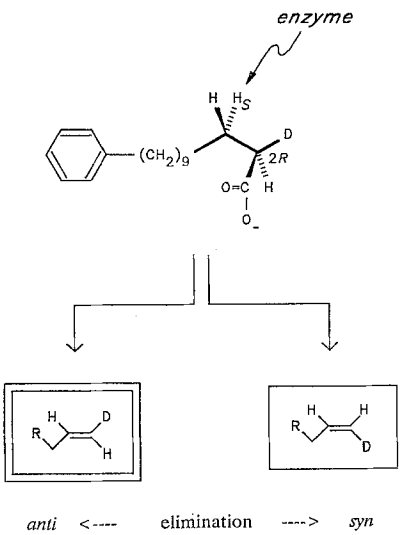


Figure 3. Evaluation of the transition state geometry of 1-alkene biosynthesis in *T. confusum*. Owing to the enantiospecifically labelled methylene group at C(2) of the precursor acid, and the known site specificity of the enzyme (specific for H_{Si}) the transition state geometry of the fragmentation reaction can be determined by the configuration of the metabolite. *Anti*-periplanar orientation of the carboxyl group and H_S at C(3) yields (*E*)-11-phenyl[1-^2H]undec-1-ene after fragmentation.

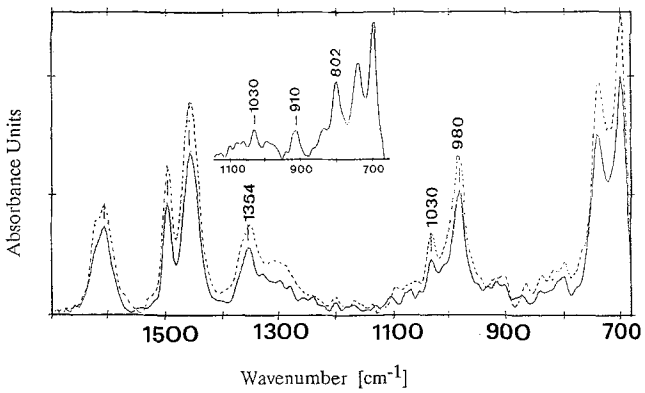
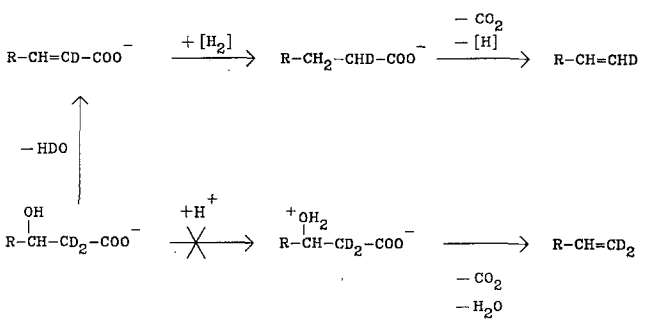


Figure 4. Gas phase infrared spectra of (*E*)- and (*Z*)-11-phenyl-[1-^{2}H]undec-1-ene. Lower traces: synthetic (*E*)-11-phenyl[1-^{2}H]undec-1-ene (——) and the metabolite (- - - -) derived from incubation of *T. confusum* with (2*R*)-12-phenyl[2-^{2}H]dodecanoic acid. Inset: (*Z*)-11-phenyl[1-^{2}H]undec-1-ene. The complete coincidence between the metabolite and the (*E*)-isomer is indicative for an *anti*-periplanar transition state geometry.

obtain sufficient material (20 µg) of the artificial metabolite. As can be seen in figure 4, the spectrum of the isolated metabolite is completely identical with that of the synthetic (*E*)-11-phenyl[1-^{2}H]undec-1-ene. This result is in accord with an *anti*-periplanar orientation of the *pro-S* C(3)-H atom and the carboxyl moiety during the fragmentation (*anti*-elimination) and corresponds to the stereochemical course of the same reaction in higher plants[8].

Evidence against 3-hydroxy intermediates from lipid metabolism. One mechanistically plausible course for this oxidative decarboxylation could be seen in an enantiospecific insertion of oxygen into the C-H bond of the *pro-(S)* C(3)-H, followed by heterolytic fragmentation of the protonated 3-hydroxy acid into H_2O, CO_2 and a 1-alkene. Such a mechanism has been postulated for the biosynthesis of vinyl groups in protoporphyrins from propionate residues of coproporphyrinogens[11], or the biosynthesis of vinylic polyacetylenes from fatty acids[12]. To exclude or confirm that such a mechanism is operative in insects, racemic 3-hydroxy-12-phenyl[2,2-$^2H_2$]-dodecanoic acid was administered to *T. confusum*. Product analysis by GLC/MS in the first instance confirms that this acid may serve as a potential precursor, but in contrast to the 11-phenyl[1,1-$^2H_2$]undec-1-ene ($M^+\cdot$ = 232 Da) derived from the nonfunctionalized 12-phenyl[2,2-$^2H_2$]dodecanoic acid, the molecular ion of the new metabolite ($M^+\cdot$ = 231 Da) now accounts for the loss of exactly one deuterium from C(2). This obvious contradiction between the two feeding experiments can be readily explained if the 3-hydroxy acid is channeled into fatty acid anabolism prior to fragmentation (scheme).



In this anabolic pathway the acid first loses water (as HDO) and (*E*)-12-phenyl[2-^{2}H]dodec-2-enoic acid is formed. After reduction to the saturated 12-phenyl-[2-^{2}H]dodecanoic acid the latter serves as the ultimate precursor for the mono deuterated 11-phenyl[1-^{2}H]-undec-1-ene. This indirect pathway was observed in plants and could be established by administration and successful conversion of the intermediary unsaturated (*E*)-12-phenyl-dodec-2-enoic acid to the corresponding 11-phenylundec-1-ene[8]. Repetition of this experiment with *T. confusum* and (*E*)-12-phenyldodec-2-enoic acid gave the same result and established once again the coincidence of 1-alkene biosynthesis in plants and insects.

Localization of 11-phenylundec-1-ene in T. confusum. If the 11-phenylundec-1-enes are produced, stored and used by the insect in the same way as the genuine hydrocarbons, the artificial metabolites should be only detectable in the tissue of the abdominal- or prothoracic glands, respectively. This is the case, and could be readily confirmed by GLC/MS analysis and solid injection of freshly-excised gland tissue. Due to the larger volume of the abdominal glands with their appended reservoirs, the tissue of a single gland was sufficient to obtain a full mass spectrum of the deuterated 11-phenylundec-1-ene. In the case of the prothoracic glands, solid injection of five glands was necessary to confirm the presence of the deuterated 11-phenylundec-1-ene. In contrast, mass spectroscopic analyses of control tissue from e.g. gut segments, hemolymph or ovaries were negative throughout, and hence the distribution of the artificial metabolites within the insect exactly corresponds to that of the genuine hydrocarbons.

Conclusions

The above results provide conclusive evidence that the biosynthesis of terminally unsaturated hydrocarbons from fatty acids in insects follows the same course as in plant metabolism. In both cases, the terminally unsaturated hydrocarbons arise from free acids as their immediate precursors, and only the *pro-(S)* C(3)-H atom and the carboxyl group of the substrate are directly involved in the biosynthetic events. Previous hypotheses, like intramolecular fragmentation of Δ^3-unsaturated acids, as proposed by Wheeler[13], or decarboxylation of appropriate intermediates of β-oxidation, are ruled out, since the complete conservation of deuterium at C(4) or C(2) of 12-phenyl[4,4-$^2H_2$, or 2,2-$^2H_2$]dodecanoic acids precludes the involvement of either Δ^3or Δ^2 unsaturated acids as intermediates. Also, heterolytic fragmentation

of 3-hydroxy acids, which has been suggested by Bohlmann[12] for the biosynthesis of terminally unsaturated polyacetylenes in plants, does not hold for the production of 1-alkenes in insects. This is sufficiently evidenced by the entirely different metabolic fate of the labelled 3-hydroxy-12-phenyl[2,2-$^2H_2$]dodecanoic acid and the successful conversion of *E*-12-phenyl[2,3-$^2H_2$]-dodec-2-enoic acid in *T. confusum*. Instead, the experimental results are framed by an enantiospecific cleavage of the C-H bond of the *pro*-(*S*) C(3)-H of the substrate acid accompanied by fragmentation into an 1-alkene and carbon dioxide via an *anti*-periplanar transition state geometry.

The general course of this fragmentation of fatty acids into 1-alkenes and carbon dioxide is also in line with the biosynthesis of some algal pheromones like e.g. undeca-1,3,5-triene and undeca-1,3,5,8-tetraene from dodeca-3,6-dienoic- or dodeca-3,6,9-trienoic acid[14], respectively. Similarly, the fragmentation of nerolidol into the odoriferous homoterpene 4,8-dimethylnona-1,3,7-triene, and butenone, which appears to be a widespread reaction in flowering plants, fits into this general scheme[15]. Hence, it appears that the oxidative fragmentation of oxygen-containing precursors (acids, secondary and tertiary alcohols) into 1-alkenes, with concomitant oxidation of a C-O single bond to a C = O bond, is a ubiquitous reaction occurring on various substrates and in different forms of life. Further work on the activation of the precursors, namely abstraction of the *pro*-(*S*) C(3)-H atom as a radical or transient insertion of oxygen into the C(3)-H bond followed by immediate fragmentation at the active center of the enzyme, is necessary to understand this type of biotransformation in more detail. Efforts in this direction are under way and will be reported in due course.

Acknowledgments. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie (Frankfurt am Main) is gratefully acknowledged. We thank Bayer AG, Leverkusen, and BASF, Ludwigshafen, for generously supplying solvents and chemicals. We also thank Dipl. Ing. K. Land, Bruker analytische Meßtechnik GmbH, Karlsruhe, for the GLC/-FTIR spectra.

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0014-4754/90/070700-05$1.50 + 0.20/0
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Circulatory and respiratory consequences of massive hemorrhage are reversed by protoveratrines

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Received 12 September 1989; accepted 2 February 1990

Summary. In a rat model of severe hypotension and respiratory depression induced by step-wise bleeding, protoveratrines cause a prompt and sustained improvement of cardiovascular and respiratory functions, both in anesthetized and in conscious animals, seemingly through a magnification of the reflex response originated by the chemoreceptors of aortic and carotid bodies. The restoration of cardiovascular function is attributable to an increase both in total peripheral resistance and cardiac output. The finding could provide the basis for a new approach to the first-aid management of massive blood losses.
Key words. Hemorrhage; hypovolemic shock; hypotension; respiratory depression; protoveratrines.

Veratrum ester alkaloids of the ceveratrum group, when administered in μg/kg doses, exert widespread effects on many body functions[1,2]. Basically, they increase reflex excitability (Bezold-Jarisch reflex and other circulatory and respiratory reflexes, etc.) and sensitize receptors involved in afferent pathways in the apparent absence of normal stimuli (sensation of warmth in the face, mouth, throat, hands, feet and perineum, without reddening of the skin; prickling and tingling sensations). They increase the sodium conductivity of the membrane of excitable cells, increase and extend the negative afterpotential, and cause repetitive discharge in response to single stimuli in nerve and muscle. Stretch- and presso-receptors of the heart and of the carotid sinus are highly sensitive to this so-called 'veratrine response'[1,2]. In hypertensive and normotensive subjects, veratrum alkaloids increase the