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Biosynthesis of sex pheromones in moths: stereochemistry of fatty alcohol oxidation in *Manduca sexta*

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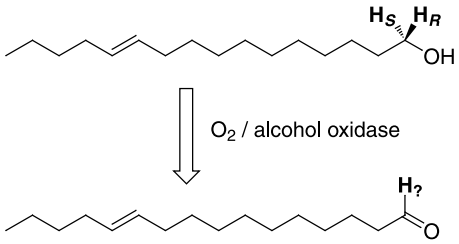
Abstract—Six chiral deuterium labelled metabolic probes, (*R*) and (*S*)-enantiomers of [1,10,10-$^2H_3$]-hexadecan-1-ol, (11*E*)-[1,10,10-$^2H_3$]-hexadec-11-en-1-ol and (11*Z*)-[1,10,10-$^2H_3$]-hexadec-11-en-1-ol, were synthesized to examine the stereospecificity of the fatty alcohol oxidase from the female pheromone gland of the tobacco hawk moth (*Manduca sexta*, Sphingidae). Both in vitro and in vivo oxidations were found to proceed by selective removal of the C1–H_R hydrogen or deuterium atom (*Re*-specificity) to yield the corresponding aldehydes. (*R*) and (*S*)-enantiomers of deuterium labelled salicyl alcohol and 2-thienyl-methanol, compounds entirely chemically diverse from the natural pheromone precursors, were also oxidised *Re*-specifically to salicylaldehyde and 2-thiophenecarb-aldehyde, respectively.

1. Introduction

Pheromones are semiochemicals produced and perceived by individuals belonging to the same species (interspecific recognition). In Lepidoptera the most important semiochemicals are sex pheromones released by females to attract conspecific males for mating.[1]

Biosynthesis of moth female sex pheromones is a part of lipid metabolism in insects, where de novo biosynthesised fatty acid precursors are desaturated and/or chain shortened by β-oxidation. The resultant CoA-esters of predominantly unsaturated acid, are transformed to hydrocarbons, alcohols, esters or aldehydes by oxidation/reduction and esterification enzymatic reactions.[2,3] Fatty acid desaturation is performed by specific Δ11-CoA-fatty acid desaturases, found only in insects.[4] The Δ11-desaturase from *Trichoplusia ni* (Lepidoptera, Tortricidae) was recently cloned and expressed in yeast.[5] These investigations of Roelofs et al. opened a way to a 'molecular era' in pheromone biosynthesis studies. The Δ11-desaturases were characterized from a stereochemical point of view by several authors.[6–8] These studies revealed that the removal of the two hydrogen atoms from prochiral C-centres is stereospecific and in the case of (*Z*)-double bond formation, it exhibits pro-(*R*) C11–H and pro-(*R*) C12–H selectivity, comparable to the more common Δ9-desaturase.[6,7] In the case of (*E*)-carbon–carbon double bond formation pro-(*R*) C11–H and pro-(*S*) C12–H stereoselectivity was observed.[8]

Another important chemical reaction is the transformation of the functional group(s) of a pheromone. This affects both, the perception by male antennae as well as the volatility of the compound. One of these transformations, the oxidation of primary alcohols to aldehydes,[9] involves removal of one of two hydrogen atoms from a prochiral $-CH_2OH$ group (Scheme 1). In Lepidoptera, there is no precedent for the stereoselectivity of this transformation, however, in Coleoptera several investigations have been reported.[10] Alcohol oxidase(s) from the leaf beetle *Phaedon armoraciae* (Coleoptera: Chrysomelidae) are responsible for the oxidation of geraniol to 8-oxo-geranial, the immediate precursor of the two iridoids chrysomelidial



Scheme 1.

Keywords: alcohols; aldehydes; biosynthesis; pheromones.

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and plagiodial. The oxidase catalyses the removal of the C1– and C8–H_R hydrogen atoms (*Re*-specificity).[11] In contrast to NADH dependent oxidoreductases from plants,[12] beetles use an oxygen-dependent enzyme. The *Re*-specificity was documented with six additional leaf beetles for related, iridoid-producing pathways.[10] The specificity is also the same with other types of substrates, e.g. salicylaldehyde, a defensive allomone of leaf beetle larvae (*Phratora vitellinae*), which is produced with *Re*-specificity from the corresponding salicylalcohol by an oxidase located in the poison gland.[10] Furthermore, the alcohol oxidase from the yeast *Candida boidinii* catalyzes the oxidation of ethanol to acetaldehyde with removal of the pro-*R* hydrogen atom (*Re*-specificity).[13]

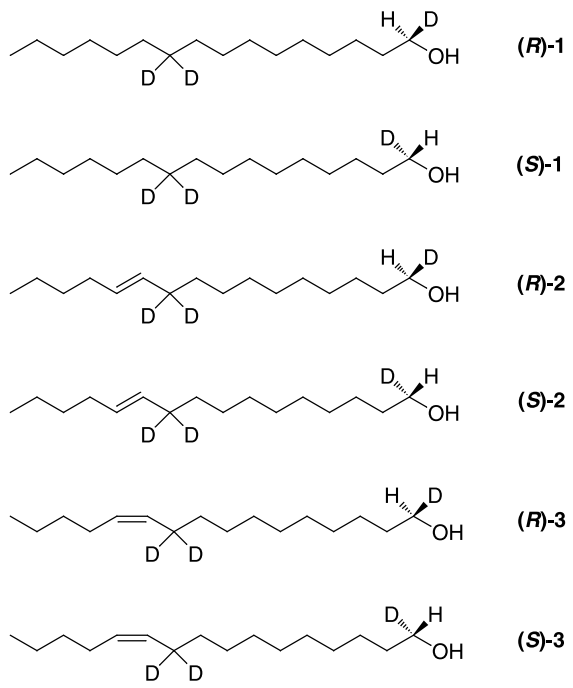
The recent observation that the alcohol oxidases catalysing the formation of the fatty aldehydes from fatty alcohols are present in the pheromone glands of several moths[14–16] has suggested the possibility of determining the stereochemistry of this transformation. For this study, the tobacco hawk moth (*Manduca sexta*, Sphingidae) was selected as a model lepidopteran species. This species is one of the most thoroughly studied insect models used in pheromone olfaction[17–21] and biosynthesis[7,22–25] research. Moreover, the basic features of the *M. sexta* pheromone alcohol oxidase producing aldehydes of various degree of unsaturation were characterised by Fang et al.[16]

The assignment of alcohol oxidase stereochemistry (Scheme 1)[†] requires the use of chiral, deuterium labelled precursors with defined stereochemistry on carbon C1. One of the pro-*R* or pro-*S* hydrogen atoms on the C1 prochiral carbon atom in the synthetic metabolic probes is replaced with a deuterium atom. The fate of those C1-attached atoms can be monitored by gas chromatography–mass spectrometry (GLC–MS).

Twelve pheromone-like compounds have been identified in solvent rinses of the *M. sexta* female pheromone gland.[26,27] To determine the stereochemistry of the oxidation of all active components of the pheromone blend would require the preparation of (*R*) and (*S*)-metabolic probes for all of them. However, the syntheses of optically active deuterated dienals and the extremely unstable[28] trienals might not be necessary. A reasonable assumption is that the stereoselectivity of the enzymatic oxidation of C16-trienols/dienols to the corresponding aldehydes is the same as for monoenic and saturated C16-compounds.[16]

Thus, six optically active [1,10,10-$^2H_3$]-C_{16}-alcohols, depicted in Scheme 2, were proposed as metabolic probes. Here we present the synthesis of the proposed probes, results of incubation experiments with them and the assignment of the stereoselectivity of the *M. sexta* alcohol oxidase.

[†] D was used as a symbol for deuterium atoms in Schemes 1–4 and plain text. In chemical names symbol ^2H was used due to IUPAC nomenclature recommendations.



Scheme 2.

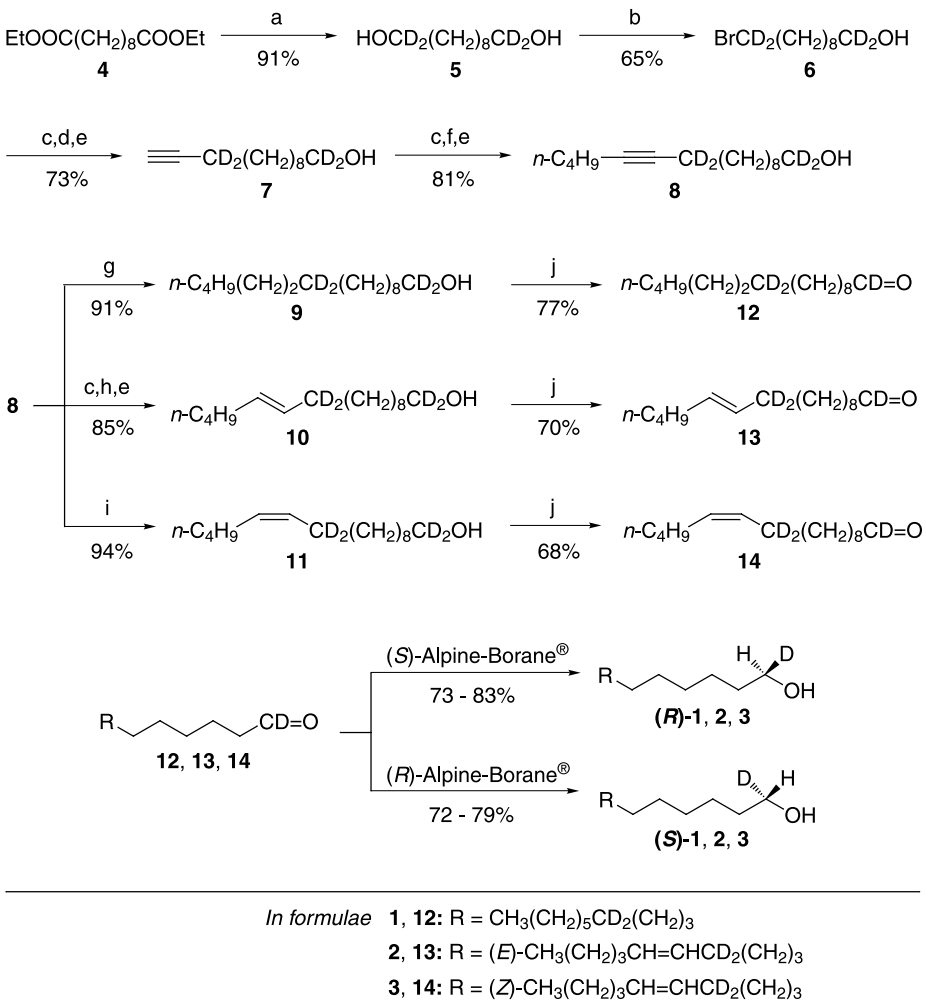
2. Results and discussion

2.1. Highly enantioselective synthesis of deuterium labelled metabolic probes

A convenient synthetic route to the chirally labelled alcohols **1**–**3** is outlined in Scheme 3. Introduction of deuterium was achieved by reduction of dimethyl sebacate (**4**) with $LiAlD_4$.[29] The product of the reaction, deuterated diol **5**, was converted to 10-bromodecan-1-ol[30] **6**, and, after protection of the hydroxy group, used for subsequent two-step alkylation of the acetylene.[31] This reaction sequence hand in hand with the necessary protection–deprotection steps afforded the key intermediate in the syntheses of desired chiral alcohols **1**–**3**, [1,1,10,10-$^2H_4$]-hexadec-11-yn-1-ol (**8**).

Hydrogenation of the akynol **8** using *tris*(triphenylphosphine)rhodium bromide (Wilkinson's catalyst[32,33]) gave hexadecanol **9**. Reduction of **8** with sodium metal in liquid ammonia[34] (with protection–deprotection of the hydroxy group) gave (*E*)-hexadecenol **10**, and finally, *cis*-semihydrogenation of **8** over P2–Ni catalyst[35] afforded (*Z*)-hexadecenol **11**. The C16-alcohols **9**–**11** were converted to the aldehydes **12**–**14** by simple oxidation with pyridinium chlorochromate (PCC).[36]

The final step of the synthesis was stereospecific reduction of the aldehydes **12**–**14** with (*S*) and (*R*)-*B*-isopinocampheyl-9-borabicyclo[3.3.1]nonanes (Alpine-Boranes®; Aldrich), which are known to be highly stereoselective reducing agents.[37,38] The enantiomeric purity of the labelled chiral alcohols **1**–**3** was determined by ^1H NMR via their (1*S*)-camphanates[39,40] according to the method of Taguchi.[38] The (*R*) and (*S*)-C_{16}-alcohols **1**–**3** were sufficiently enantiomerically pure (92–96%; see Table 1) for the experiments with pheromone alcohol oxidase.



In formulae **1**, **12:** R = $CH_3(CH_2)_5CD_2(CH_2)_3$

2, **13:** R = (*E*)-$CH_3(CH_2)_3CH{=}CHCD_2(CH_2)_3$

3, **14:** R = (*Z*)-$CH_3(CH_2)_3CH{=}CHCD_2(CH_2)_3$

Scheme 3. (a) $LiAlD_4$/ether; (b) HBr/benzene, reflux 8 h; (c) 3,4-dihydro-2*H*-pyran, PPTS/CH_2Cl_2; (d) $LiHC_2/NH_3$ (*liquid*), DMSO; (e) Dowex 50W $H^{\oplus}$/MeOH; (f) (1) BuLi/THF, HMPA; (2) *n*-BuI/THF; (g) H_2, $Rh[Ph_3P]_3Br$/benzene; (h) Na/NH_3 (liquid), ether; (i) H_2, P2–Ni/EtOH; (j) PCC, $NaOAc/CH_2Cl_2$.

2.2. In vitro and in vivo oxidation of deuterium labelled metabolic probes

In vitro oxidation experiments were carried out using 0.1% solutions of the chiral C16-alcohols **1**–**3** in hexane. The solution of the substrate was shaken with a small volume of phosphate buffer (pH 7.2) containing *M. sexta* pheromone gland tissue. Mass spectroscopic analysis of the products unambiguously demonstrated the stereospecificity of the alcohol oxidase in *M. sexta*. The determination of the

Table 1. In vitro oxidations of labelled chiral alcohols by *M. sexta* pheromone gland alcohol oxidase

Chiral alcohol	ee (%)	Conversion[a,b] (%)	Aldehyde dominant ions[c] (*m/z* and rel. abundance[b] in%)			Oxidase stereospecificity
			$[M+1]^+$	$[M-17]^+$	$[M+57]^+$	
(*R*)-**1**	96	65±12	243 (100)	225 (10)	299 (33)	*Re*
(*S*)-**1**	95	70±8	244 (100)	226 (18)	300 (10)	*Re*
(*R*)-**2**	93	77±10	241 (100)	223 (70)	297 (31)	*Re*
(*S*)-**2**	94	68±11	242 (100)	224 (74)	298 (12)	*Re*
(*R*)-**3**	93	94±9	241 (100)	223 (66)	297 (47)	*Re*
(*S*)-**3**	92	91±15	242 (100)	224 (83)	298 (11)	*Re*
(*R*)-**16**	>99	90±7	122 (100)	104 (54)	178 (19)	*Re*
(*S*)-**17**	>99	88±14	127 (100)	110 (60)	183 (21)	*Re*
(*R*)-**18**	>99	100±0	113 (100)	95 (51)	169 (28)	*Re*
(*S*)-**18**	>99	100±0	114 (100)	96 (44)	170 (25)	*Re*

[a] Conversion of chiral alcohols to corresponding aldehydes was determined by GLC.
[b] Average value from GLC–MS analyses of three experiments.
[c] GLC–MS (positive CI; 2-methylpropane) of products of the chiral alcohols oxidations.

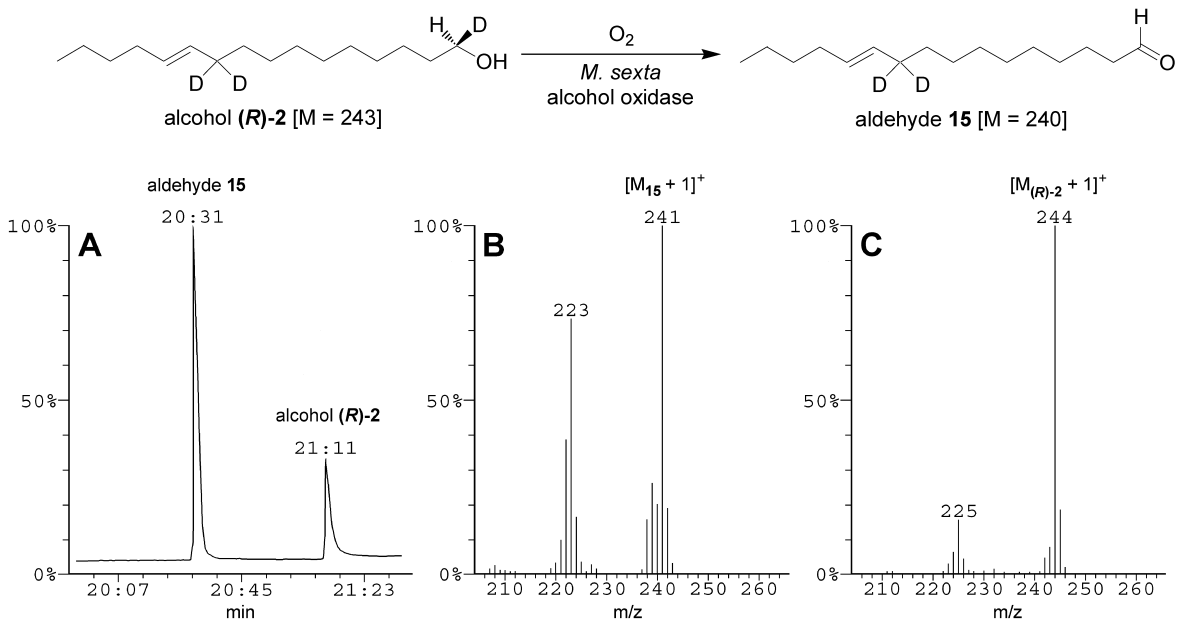


Figure 1. In vitro enzymatic oxidation of (1*R*,11*E*)-[1,10,10-$^2H_3$]-hexadec-11-en-1-ol, (*R*)-**2**. (A): section of the chromatogram obtained by GLC–MS (CI) analysis of the reaction mixture after 48 h of incubation. (B): section of CI-MS spectra of the aldehyde **15** with dominant $[M-17]^+$and $[M+1]^+$ions. (C): section of CI-MS spectra of the chiral labelled alcohol (*R*)-**2** with dominant $[M-17]^+$and $[M+1]^+$ions.

enzyme specificity can be illustrated by the oxidations of (1*R*,11*E*)-[1,10,10-$^2H_3$]-hexadec-11-en-1-ol [(*R*)-**2**] and (1*S*,11*E*)-[1,10,10-$^2H_3$]-hexadec-11-en-1-ol [(*S*)-2]. Alcohol (*R*)-**2** was oxidised to aldehyde **15** in vitro (Fig. 1) with 77% conversion, while alcohol (*S*)-**2** was oxidised to aldehyde **13** (Fig. 2) with 68% conversion. The identities of the oxidation products followed from their CI-MS spectra. Both aldehydes displayed dominant (100%) pseudo-molecular ions $[M+1]^+$ accompanied by intense $[M-17]^+$ and $[M+57]^+$ ions. Incubations of pheromone gland tissue with (*R*)-**2** resulted in aldehyde **15** (100% $[M+1]^+$ ion at *m/z* 241 Da, $[M-17]^+$ ion at *m/z* 223 Da and $[M+57]^+$ ion at *m/z* 297 Da). This indicates the loss of the deuterium atom from the labelled precursor (*R*)-**2**. Incubation of (*S*)-**2** resulted in aldehyde **13** with the pseudo-molecular ion $[M+1]^+$ at *m/z* 242 Da, $[M-17]^+$ ion at *m/z* 224 Da and finally $[M+57]^+$ ion at *m/z* 298 Da. The data are consistent with a loss of one hydrogen atom from the alcohol (*S*)-**2**.

In vitro experiments with the metabolic probes (*R*)-**1**/(*S*)-**1** and (*R*)-**3**/(*S*)-**3** gave identical results. Thus, the alcohol oxidase of the *M. sexta* pheromone gland has been shown to remove selectively the C1–H_R hydrogen/deuterium atom of

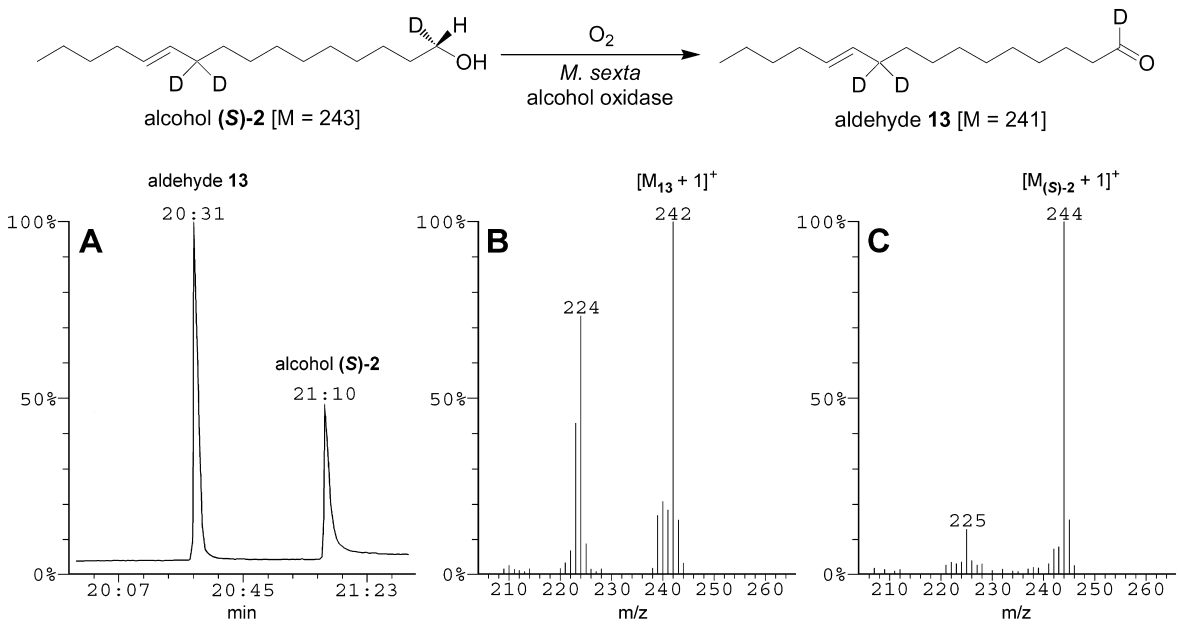
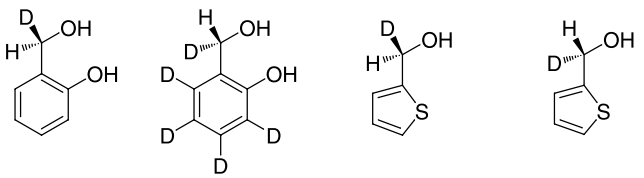


Figure 2. In vitro enzymatic oxidation of (1*S*,11*E*)-[1,10,10-$^2H_3$]-hexadec-11-en-1-ol, (*S*)-**2**. (A): section of the chromatogram obtained by GLC–MS (CI) analysis of the reaction mixture after 48 h of incubation. (B): section of CI-MS spectra of the aldehyde **13** with dominant $[M-17]^+$and $[M+1]^+$ions. (C): section of CI-MS spectra of the chiral labelled alcohol (*S*)-**2** with dominant $[M-17]^+$and $[M+1]^+$ ions.



Scheme 4.

chiral deuterium labelled alcohols during oxidation to the corresponding aldehydes (Table 1). The *Re*-stereospecificity of the alcohol oxidase was also observed by in vivo incubations with saturated alcohols (*R*)-**1** and (*S*)-**1** carried out on *M. sexta* females. The GLC–MS analyses of the hexane extracts of excised pheromone glands gave identical results to the corresponding above described in vitro experiments.

Fang et al. demonstrated[16] that the oxidase of *M. sexta* converts alcohols of different chain length (C14–C17). We were able to show that the specificity of this oxidase is even broader and it can oxidise other primary alcohols, including aromatic, allylic or heterocyclic compounds.[41] This earlier work prompted in vitro incubation experiments with chiral deuterium labelled salicylalcohol (*R*)-**16**/(*S*)-**17** and 2-thienylmethanol (*R*)-**18**/(*S*)-**18** (Scheme 4; syntheses in Refs. 10,41). Although the structures of these compounds are different from natural pheromone precursors, namely saturated and unsaturated C16-fatty alcohols, the enzyme produced the corresponding aldehydes and displayed *Re*-stereospecificity in all cases (Table 1).

It is interesting, that all examined alcohol oxidases from yeasts,[13] plants[12] or leaf beetles[10,11] remove selectively the pro-H_R enantiotropic hydrogen atoms of the prochiral $-CH_2OH$ groups during the oxidation of substrates to aldehydes. The *Re*-stereospecificity of these enzymes is not dependent on their basic features (O_2 or $NADH^+$ oxidation system, substrate specificity, etc.). The question of whether *Re*-stereospecificity is a common attribute of all lepidopteran alcohol oxidases, or is limited to *M. sexta* remains to be established. The herein prepared metabolic probes can be used to determine the stereoselectivity of alcohol oxidases in other lepidopteran species[1] (for example: diamondback moth (*Plutella xylostella*; Plutellidae), Eastern spruce budworm (*Choristoneura fumiferana*; Tortricidae), corn earworm (*Heliothis virescens*; Noctuidae), and tomato fruitworm (*Heliothis zea*; Noctuidae)] covering a broader phylogenetic range. Investigations into this question are in progress and the first results will be reported soon.

3. Conclusions

Using alkyne/borane chemistry we prepared a set of chiral deuterium labelled metabolic probes **1**–**3**, which were used for both in vitro and in vivo determination of the stereospecificity of *M. sexta* pheromone alcohol oxidase. The data clearly established *Re*-stereospecificity for the oxidation process. In vitro oxidations of non-natural chiral aromatic/heterocyclic alcohols (*R*)-**16**, (*S*)-**17**, (*R*)-**18** and (*S*)-**18** gave the same results, demonstrating that this stereospecificty is intrinsically linked to the oxidation process.

4. Experimental

4.1. Chemical Synthesis

NMR spectra were determined in $CDCl_3$ solutions on a Varian UNITY-500 spectrometer operating at 499.5 MHz for ^1H and at 125.7 MHz for ^{13}C NMR, respectively. Chemical shifts are expressed in δ (ppm) scale relative to tetramethylsilane for ^1H and relative to $CDCl_3$ signal (77.00 ppm) for ^{13}C NMR, respectively. Coupling constants are reported in Hz. IR spectra (wavenumbers in cm^{-1}) were recorded on a Bruker Equinox 55 FT-IR spectrometer in CCl_4 solutions. GLC–GLC–MS (EI; 70 eV) analyses were performed on a Finnigan GCQ equipped with J and W Scientific (DB-5 capillary column 30 m×0.25 mm, 0.25 μm film thickness; with helium (linear velocity 30 mL s^{-1}) as carrier gas). High-resolution MS (EI) data were obtained using a Micromass MasSpec 2. GLC–MS (CI: chemical ionisation in the positive ion mode with 2-methylpropane) analyses were performed using a Hewlett Packard HP6890 gas chromatograph equipped with a J and W Scientific DB-5 capillary column (30 m×0.25 mm, 0.25 μm film thickness; with helium (linear velocity 30 mL s^{-1}) as carrier gas) interfaced to a Micromass MasSpec 2. Flash chromatography: Merck 60 silica gel (0.040–0.063 mm). Alcohols **1**–**3** were chromatographed on Merck 60 silica gel impregnated with 5% silver nitrate for complete removal of boron contaminants (from Alpine-Boranes®).[42]

All reactions were run in oven-dried glassware under argon. Tetrahydrofuran (THF), ether and benzene were distilled from sodium benzophenone ketyl under argon. Dichloromethane was distilled from calcium hydride and stored over molecular sieves. Hexamethylphosphoramide (HMPA) and dimethylsulfoxide (DMSO) were dried over molecular sieves. All other chemicals were used as purchased. NMR spectra and high-resolution MS analyses of synthetic compounds (>98% according to GLC) were fully consistent with the proposed structures.

4.1.1. [1,1,10,10-$^2H_4$]-Decane-1,10-diol[29] (5). Lithium aluminium deuteride ($LiAlD_4$; 7.39 g, 176 mmol) was suspended in ether (300 mL). Dimethyl sebacate (**4**; 36.82 g, 160 mmol) in ether (50 mL) was added dropwise over 1 h with stirring at 0°C. After refluxing for 5 h, the mixture was decomposed with ice-cold water (40 mL) and 25% sulphuric acid (100 mL). The organic phase was separated, filtered through Celite and extracted with ether (4×75 mL). The combined organic extracts were washed with brine (2×100 mL) and dried ($MgSO_4$). Removal of the solvent in vacuo afforded crude diol which was recrystallised from benzene to give pure product **5** (white crystalline solid; 25.94 g, 91%), mp 72–73°C (lit., 72.5°C).[29] Spectroscopic data were consistent with the literature.[29]

4.1.2. 10-Bromo-[1,1,10,10-$^2H_4$]-decan-1-ol (6). Hydrobromic acid (46%; 16.4 mL, 140 mmol) was added to a

solution of diol **5** (25.0 g, 140 mmol) in benzene (300 mL), and the mixture was heated under reflux for 8 h while trapping the reaction water using a Dean–Stark water separator. The mixture was washed with 20% NaOH (150 mL), 10% hydrochloric acid (150 mL), water (2×200 mL), brine (200 mL) and dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified by flash chromatography (15% ethyl acetate in light petroleum) to give 21.91 g (65%) of bromo alcohol **6** as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.28–1.46 (12H, m, CH_2[3–8]), 1.55 (2H, bt, J=7.8 Hz, CH_2[2]), 1.84 (2H, bt, J=7.3 Hz, CH_2[9]). ^{13}C NMR ($CDCl_3$) δ: 25.65 (t), 28.08 (t), 28.72 (t), 29.21 (m, J_{CD}=4.4 Hz, C_2), 29.33 (t), 29.35 (t), 29.45 (t), 32.57 (t), 33.50 (m, J_{CD}=23.9 Hz, C_{10}), 62.29 (t, J_{CD}=21.5 Hz, C_1). FT-IR (CCl_4, cm^{-1}): ν (OH) 3638m; ν_{AS} (CD_2) 2264w, 2196w; ν_S (CD_2) 2161w, 2091w; ν_S (C–O)+γ_S (CD_2) 961s, 1165m; ν (C–Br) 609w, 544m. HRMS: for $C_{10}H_{17}D_4^{79}BrO$ calculated 240.1022; found 240.1020.

4.1.3. [1,1,10,10-$^2H_4$]-Dodec-11-yn-1-ol (7). 3,4-Dihydro-2*H*-pyran (8.0 g, 95.0 mmol) was added dropwise to a stirred solution of bromo alcohol **6** (21.7 g, 90.0 mmol) and pyridinium *p*-toluenesulfonate (100 mg, 0.35 mmol) in CH_2Cl_2 (250 mL) at 0°C. After stirring for 8 h at 0°C, the mixture was diluted with ether (250 mL), washed with a saturated solution of $NaHCO_3$ (2×100 mL), brine (100 mL) and dried over K_2CO_3. The solvents were evaporated and the resulting THP-protected bromo alcohol (29.3 g) was added dropwise to a stirred suspension of freshly prepared lithium acetylide (120 mmol; the preparation is described in lit.[31]) in liquid ammonia (400 mL) and anhydrous DMSO (250 mL). After stirring for 4 h, the ammonia was allowed to evaporate and brine (800 mL) was added. The mixture was extracted with hexane (5×150 mL) and the combined extracts dried over $MgSO_4$. Evaporation of the solvents furnished 24.2 g of a red oil that was dissolved in methanol (300 mL) and treated with Dowex 50 W ion-exchange resin (H^+ form, 10 g) for 24 h. The resin was removed by filtration and the solvent removed in vacuo. Purification of the residue by flash chromatography (15% ethyl acetate in light petroleum) gave 12.20 g (73%) of alkynol **7** as a colourless oil. ^{1}H NMR ($CDCl_3$) δ: 1.27–1.42 (12H, m, CH_2[3–8]), 1.51 (2H, m, CH_2[9]), 1.55 (2H, bt, J=7.8 Hz, CH_2[2]), 1.93 (1H, s, CH). ^{13}C NMR ($CDCl_3$) δ: 17.8 (m, J_{CD}=20.0 Hz, C_{10}), 25.7 (t, C_3), 28.3 (t), 28.7 (t), 29.0 (t), 2×29.4 (t), 29.5 (t), 32.6 (t), 62.3 (t, J_{CD}=22.0 Hz, C_1), 68.0 (s, C_{12}), 84.7 (s, C_{11}). FT-IR (CCl_4, cm^{-1}): ν (OH) 3638m; ν (≡CH) 3315s; ν (C≡C) 2120w; δ (≡CH) 633s; ν_{AS} (CD_2) 2198w; ν_S (CD_2) 2096m; ν_S (C–O)+γ_S (CD_2) 961m, 1168m. HRMS: for $C_{12}H_{18}D_4O$ calculated 186.1920; found 186.1922.

4.1.4. [1,1,10,10-$^2H_4$]-Hexadec-11-yn-1-ol (8). 3,4-Dihydro-2*H*-pyran (5.89 g, 70 mmol) was added dropwise to a stirred solution of alcohol **7** (12.1 g, 65 mmol) and pyridinium *p*-toluenesulfonate (100 mg, 0.35 mmol) in CH_2Cl_2 (200 mL) at 0°C. After stirring for 6 h at the same temperature, the mixture was diluted with ether (250 mL), washed with a saturated solution of $NaHCO_3$ (2×100 mL), brine (100 mL) and dried over K_2CO_3. The solvents were evaporated and the THP-protected alkynol (17.6 g) was dissolved in THF (60 mL) and HMPA (20 mL). The alkyne was deprotonated at −50°C with *n*-butyllithium in hexanes (2.5 M; 28 mL, 70 mmol) over period of 30 min. The mixture was warmed to 0°C over a period of 30 min, stirred at this temperature for 30 min, and kept at rt for 1 h. The mixture was cooled to −20°C and a solution of 1-iodobutane (12.9 g, 70 mmol) in THF (20 mL) was added over a period 30 min. The red reaction mixture was first stirred at 0°C for 5 h, and then at rt overnight. The reaction was quenched by saturated NH_4Cl solution (300 mL). Extraction with hexane:ether (2:1; 3×100 mL), drying ($MgSO_4$) and removal of solvents furnished 16.2 g of a red oil that was dissolved in methanol (200 mL) and treated with Dowex 50W ion-exchange resin (H^+ form, 20 g) for 24 h. The ion-exchange resin was filtered off and the solvent removed in vacuo. Purification of the residue by flash chromatography (15% ethyl acetate in light petroleum) gave 12.76 g (81%) of alkynol **8** as a colourless oil. ^{1}H NMR ($CDCl_3$) δ: 1.26 (3H, t, J=7.2 Hz, CH_3), 1.28–1.50 (18H, m, CH_2[3–9,14,15]), 1.55 (2H, bt, J=7.8 Hz, CH_2[2]), 2.13 (2H, t, J=6.9 Hz, CH_2[13]). ^{13}C NMR ($CDCl_3$) δ: 13.6 (q, C_{16}), 18.1 (m, J_{CD}=19.0 Hz, C_{10}), 18.4 (t, C_{13}), 21.9 (t, C_{15}), 25.7 (t), 28.8 (t), 29.0 (t), 29.1 (t), 29.4 (t), 29.4 (t), 29.5 (t), 31.3 (t), 32.59 (t), 62.4 (m, J_{CD}=21.5 Hz, C_1), 80.1 (s, C_{11}), 80.2 (s, C_{12}). FT-IR (CCl_4, cm^{-1}): ν (OH) 3638m; ν_{AS} (CD_2) 2194w; ν_S (CD_2) 2096w; ν_S (C–O)+γ_S (CD_2) 960s, 1164m; ν_{AS} (CH_3) 2958s. HRMS: for $C_{16}H_{26}D_4O$ calculated 242.2548; found 242.2546.

4.1.5. [1,1,10,10-$^2H_4$]-Hexadecan-1-ol (9). A solution of alkynol **8** (1.45 g, 6.0 mmol) in degassed benzene (5 mL) was added to a solution of $Rh(Ph_3P)_3Br$ (100 mg, 0.1 mmol) in the same solvent (150 mL) and hydrogenated with stirring (ultrasonic bath) at r.t. for 3 h. The solvent was evaporated and the residue was purified by flash chromatography (15% ethyl acetate in light petroleum) to give 1.35 g (91%) of alkanol **9** as a white crystalline solid (mp 47–48.5°C). ^{1}H NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz, CH_3), 1.21–1.38 (24H, m, CH_2[3–9,11–15]), 1.55 (2H, bt, J=7.2 Hz, CH_2[2]). ^{13}C NMR ($CDCl_3$) δ: 14.1 (q, C_{16}), 22.6 (t, C_{15}), 25.6 (t), 28.8 (tt, J_{CD}=19.1 Hz, C_{10}), 29.3 (t), 3×29.4 (t), 5×29.6 (t), 31.9 (t), 32.46 (t), 62.3 (tt, J_{CD}=21.6 Hz, C_1). FT-IR (CCl_4, cm^{-1}): ν (OH) 3639w; ν_{AS} (CD_2) 2179w; ν_S (CD_2) 2096w; ν_S (C–O)+γ_S (CD_2) 960w, 1166w; ν_{AS} (CH_3) 2956m. HRMS: for $C_{16}H_{30}D_4O$ calculated 246.2860; found 246.2858.

4.1.6. (11*E*)-[1,1,10,10-$^2H_4$]-Hexadec-11-en-1-ol (10). 3,4-Dihydro-2*H*-pyran (0.59 g, 7.0 mmol) was added dropwise to a stirred solution of alcohol **8** (1.45 g, 6.0 mmol) and pyridinium *p*-toluenesulfonate (20 mg, 70 μmol) in CH_2Cl_2 (50 mL) at 0°C. After stirring for 8 h at the same temperature, the mixture was diluted with ether (100 mL), washed with a saturated solution of $NaHCO_3$ (2×50 mL), brine (50 mL) and dried over K_2CO_3. The solvents were evaporated, the O-protected alcohol **8** (2.0 g) was diluted with ether (80 mL), and added dropwise to a stirred freshly prepared solution of sodium (0.46 g, 20.0 mmol) in liquid ammonia (200 mL). After stirring for 8 h, ammonia was allowed to evaporate and the residue treated with brine (200 mL). The mixture was extracted with ether (4×75 mL) and the combined extracts dried over $MgSO_4$. Evaporation of the solvents furnished 1.9 g of a yellow oil that was dissolved in methanol (50 mL) and treated with Dowex 50 W ion-exchange resin (H^+ form, 1 g) for 24 h. The ion-exchange resin was filtered off and the solvent removed in

vacuo. Purification of the residue by flash chromatography (15% ethyl acetate in light petroleum) gave 1.25 g (85%) of (*E*)-alkenol **10** (98% isomeric purity, GLC) as a colourless oil. ^{1}H NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.2 Hz, CH_3), 1.24–1.36 (18H, m, CH_2[3–9,14,15]), 1.55 (2H, bt, J=7.3 Hz, CH_2[2]), 1.95–1.99 (2H, m, CH_2[13]), 5.34–5.42 (2H, m, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 13.9 (q, C_{16}), 22.2 (t, C_{15}), 25.7 (t), 26.8 (tt, J_{CD}=19.2 Hz, C_{10}), 29.4 (t), 4×29.5 (t), 29.6 (t), 31.9 (t, C_2), 32.3 (t), 32.6 (t), 62.34 (tt, J_{CD}=21.4 Hz, C_1), 130.3 (d, C_{11}), 130.4 (d, C_{12}). FT-IR (CCl_4, cm^{-1}): ν(OH) 3638m; ν_{AS} (=CH) 3016m; ν(C=C) 1665w; γ(=CH) 970s; ν_{AS} (CD_2) 2187w; ν_S (CD_2) 2095w; ν_S (C–O)+γ_S (CD_2) 960s, 1167w; ν_{AS} (CH_3) 2957s. HRMS: for $C_{16}H_{28}D_4O$ calculated 244.2704; found 244.2708.

4.1.7. (11*Z*)-[1,1,10,10-$^2H_4$]-Hexadec-11-en-1-ol (11). 1,2-Diaminoethane (40 μL) and alkynol **8** (1.45 g, 6.0 mmol) were added to a suspension of P2–Ni (prepared from 50 mg of nickel(II) acetate and 200 μL of 1 M $NaBH_4$ in EtOH) in ethanol 30 mL and hydrogenated with stirring at rt. The progress of the hydrogenation was monitored by GLC–MS. The usual workup and flash chromatography (15% ethyl acetate in light petroleum) of the crude product afforded 1.37 g (94%) of (*Z*)-alkenol **11** (98.5% isomeric purity, GLC) as a colourless oil. ^{1}H NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.1 Hz, CH_3), 1.22–1.38 (18H, m, CH_2[3–9,14,15]), 1.55 (2H, bt, J=7.9 Hz, CH_2[2]), 1.97–2.01 (2H, m, CH_2[13]), 5.32–5.38 (2H, m, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 14.00 (q, C_{16}), 22.3 (t, C_{15}), 25.7 (t), 26.5 (tt, J_{CD}=19.2 Hz, C_{10}), 26.9 (t), 29.2 (t), 29.4 (t), 29.5 (t), 3×29.6 (t), 32.0 (t, C_2), 32.6 (t), 62.35 (tt, J_{CD}=21.5 Hz, C_1), 129.5 (d, C_{11}), 129.9 (d, C_{12}). FT-IR (CCl_4, cm^{-1}): ν (OH) 3638w; ν_{AS} (=CH) 3005m; ν (C=C) 1651w; β (=CH) 1401w; γ (=CH) 707w; ν_{AS} (CD_2) 2193w; ν_S (CD_2) 2097w; ν_S (C–O)+γ_S (CD_2) 961m, 1162m; ν_{AS} (CH_3) 2957s. HRMS: for $C_{16}H_{28}D_4O$ calculated 244.2704; found 244.2702.

4.1.8. [1,10,10-$^2H_3$]-Hexadecanal (12). Alkanol **9** (1.23 g, 5.0 mmol) was injected into a stirred suspension of pyridinium chlorochromate (PCC; 1.51 g, 7.0 mmol) and anhydrous sodium acetate (200 mg) in dichloromethane (10 mL). The mixture was stirred at rt for 90 min, poured into ether (50 mL) and filtered through a combined layer of neutral alumina/charcoal/Celite. Evaporation of solvents and flash chromatography (3% diethyl ether in light petroleum) afforded 937 mg (77%) of aldehyde **12** as a colourless oil. ^{1}H NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz, CH_3), 1.22–1.35 (24H, m, CH_2[3–9,11–15]), 2.41 (2H, t, J=7.4 Hz, CH_2[2]), 1.63 (2H, m, CH_2[3]). ^{13}C NMR ($CDCl_3$) δ: 14.10 (q, C_{16}), 22.1 (t), 22.7 (t), 28.8 (m, J_{CD}=14.2 Hz, C_{10}), 29.2 (t), 3×29.4 (t), 2×29.5 (t), 3×29.6 (t), 31.9 (t), 43.7 (tt, J_{CD}=3.7 Hz, C_2), 202.7 (td, J_{CD}=26.1 Hz, C_1). FT-IR (CCl_4, cm^{-1}): ν (C=O) 1719s; β(OCD) 1094w; ν_{AS} (CD_2) 2178w; ν_S (CD_2) 2100w; ν(C–D) 2063w; ν_{AS} (CH_3) 2956s. HRMS: for $C_{16}H_{29}D_3O$ calculated 243.2641; found 243.2640.

4.1.9. (11*E*)-[1,10,10-$^2H_3$]-Hexadec-11-enal (13). Aldehyde **13** (845 mg, 70%; colourless oil) was prepared from (*E*)-alkenol **10** (1.22 g, 5.0 mmol) using PCC (1.51 g, 7.0 mmol) as described for **12**. ^{1}H NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz, CH_3), 1.22–1.38 (18H, m, CH_2[3–9,14,15]), 1.95–2.00 (2H, m, CH_2[13]), 2.40 (2H, bt, J=7.2 Hz, CH_2[2]), 5.36–5.43 (2H, m, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 13.9 (q, C_{16}), 22.1 (t, C_{15}), 22.5 (t), 26.7 (tt, J_{CD}=19.1 Hz, C_{10}), 2×29.4 (t), 29.5 (t), 3×29.6 (t), 29.7 (t), 32.1 (t), 43.8 (tt, J_{CD}=21.2 Hz, C_2), 130.2 (d, C_{11}), 130.3 (d, C_{12}), 202.7 (t, J_{CD}=25.9 Hz, C_1). FT-IR (CCl_4, cm^{-1}): ν (C=O) 1719s; β (OCD) 1094w; ν_{AS} (=CH) 3016w; ν (C=C) 1667w; γ (=CH) 971m; ν_{AS} (CD_2) 2187w; ν_S (CD_2) 2103w; ν (C–D) 2063w; ν_{AS} (CH_3) 2957s. HRMS: for $C_{16}H_{27}D_3O$ calculated 241.2485; found 241.2485.

4.1.10. (11*Z*)-[1,10,10-$^2H_3$]-Hexadec-11-enal (14). Aldehyde **14** (821 mg, 68%; colourless oil) was prepared from (*Z*)-alkenol **11** (1.22 g, 5.0 mmol) using PCC (1.51 g, 7.0 mmol) as described for **12**. ^{1}H NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.1 Hz, CH_3), 1.22–1.36 (16H, m, CH_2[3–9,14]), 1.62 (2H, m, CH_2[15]), 2.41 (2H, bt, J=7.3 Hz, CH_2[2]), 2.0–2.04 (2H, m, CH_2[13]), 5.33 (1H, bd, J=11.2 Hz, CH=CH), 5.36 (1H, dt, J=2×5.8, 11.2 Hz, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 14.00 (q, C_{16}), 22.0 (t), 22.3 (t), 26.4 (tt, J_{CD}=19.0 Hz, C_{10}), 26.9 (t), 2×29.2 (t), 29.3 (t), 2×29.4 (t), 29.5 (t), 32.0 (t, C_2), 43.7 (tt, J_{CD}=3.7 Hz, C_2), 129.7 (d, C_{11}), 129.9 (d, C_{12}), 202.6 (t, J_{CD}=25.9 Hz, C_1). FT-IR (CCl_4, cm^{-1}): ν (C=O) 1719s; β (OCD) 1094w; ν_{AS} (=CH) 3006w; ν (C=C) 1653w; β (=CH) 1404w; γ (=CH) 708w; ν_{AS} (CD_2) 2190w; ν_S (CD_2) 2102w; ν(C–D) 2064w; ν_{AS} (CH_3) 2957s. HRMS: for $C_{16}H_{27}D_3O$ calculated 241.2485; found 241.2485.

4.1.11. (1*R*)-[1,10,10-$^2H_3$]-Hexadecan-1-ol [(*R*)-1]. (*S*)-Alpine-Borane® (0.5 M solution in THF; 7.5 mL, 3.73 mmol) was added to a solution of aldehyde **12** (300 mg, 1.23 mmol) in THF (10 mL). The mixture was stirred for 72 h at rt and then cooled in an ice-bath. Acetaldehyde (300 μL) was added to the mixture, which was then stirred at rt for 1 h to decompose excess borane and finally evaporated in vacuo. The residue was dissolved in ether (50 mL), ethanolamine (300 μL) was added at rt and stirring was continued for 1 h. The insoluble precipitate was filtered off, and the organic layer was washed with 1 M HCl (2×25 mL), a saturated solution of $NaHCO_3$ (2×25 mL), brine (2×25 mL) and dried over $MgSO_4$. Evaporation of the solvents gave an oily residue, which was extracted with hexane. The extract was evaporated and the residue purified by flash chromatography ($AgNO_3$ impregnated[42] silica gel; 15% ethyl acetate in light petroleum) to give 251 mg (83%) of the target alcohol (*R*)-**1** as a white crystalline solid (mp 48–49°C). ^{1}H NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz, CH_3), 1.22–1.38 (24H, m, CH_2[3–9,11–15]), 1.56 (2H, bq, J=7.1 Hz, CH_2[2]), 3.62 tt (1H, J=2×1.5, 2×6.6 Hz, CHD). ^{13}C NMR ($CDCl_3$) δ: 14.1 (q, C_{16}), 22.7 (t, C_{15}), 25.7 (t), 28.8 (tt, J_{CD}=18.8 Hz, C_{10}), 2×29.4 (t), 2×29.5 (t), 4×29.6 (t), 29.7 (t), 31.9 (t), 32.7 (t), 62.7 (tt, J_{CD}=21.7 Hz, C_1). FT-IR (CCl_4, cm^{-1}): ν (OH) 3639w; ν (C–OH) 1063w; ν_{AS} (CD_2) 2176w; ν(C–D) 2148w; ν_S (CD_2) 2101w; ν_{AS} (CH_3) 2956m. HRMS: for $C_{16}H_{31}D_3O$ calculated 245.2798; found 245.2795.

4.1.12. (1*S*)-[1,10,10-$^2H_3$]-Hexadecan-1-ol [(*S*)-1]. Alcohol (*S*)-**1** (233 mg, 77%; white crystalline solid, mp 47.5–49°C) was prepared from aldehyde **12** (300 mg, 1.23 mmol) and (*R*)-Alpine-Borane® (0.5 M solution in THF; 7.5 mL,

3.73 mmol) following the procedure for (*R*)-**1**. NMR and FT-IR data are identical with (*R*)-**1**. HRMS: for $C_{16}H_{31}D_3O$ calculated 245.2798; found 245.2797.

4.1.13. (1*R*,11*E*)-[1,10,10-$^2H_3$]-Hexadec-11-en-1-ol [(*R*)-2]. Alcohol (*R*)-**2** (240 mg, 80%; colourless oil) was prepared from aldehyde **13** (300 mg, 1.24 mmol) and (*S*)-Alpine-Borane® (0.5 M solution in THF; 7.5 mL, 3.73 mmol) following the procedure for (*R*)-**1**. δ: 0.89 (3H, t, J=7.1 Hz, CH_3), 1.23–1.38 (18H, m, CH_2[3–9,14,15]), 1.55 (2H, m, CH_2[2]), 1.94–1.99 (2H, m, CH_2[13]), 5.37–5.40 (2H, m, CH=CH), 3.62 tt (1H, J=2×1.5, 2×6.6 Hz, CHD). ^{13}C NMR ($CDCl_3$) δ: 14.0 (q, C_{16}), 22.2 (t, C_{15}), 25.7 (t), 26.9 (tt, J_{CD}=19.3 Hz, C_{10}), 29.1 (t), 29.4 (t), 3×29.5 (t), 29.6 (t), 31.8 (t, C_2), 32.3 (t), 32.7 (t), 62.7 (tt, J_{CD}=21.5 Hz, C_1), 130.3 (d, C_{11}), 130.4 (d, C_{12}). FT-IR (CCl_4, cm^{-1}): ν(OH) 3638w; ν(C–OH) 1067m; ν_{AS} (=CH) 3016w; ν(C=C) 1666w; γ(=CH) 971s; ν_{AS} (CD_2) 2182w; ν(C–D) 2148w; ν_S (CD_2) 2099w; ν_{AS} (CH_3) 2957s. HRMS: for $C_{16}H_{19}D_3O$ calculated 243.2641; found 243.2642.

4.1.14. (1*S*,11*E*)-[1,10,10-$^2H_3$]-Hexadec-11-en-1-ol [(*S*)-2]. Alcohol (*S*)-**2** (217 mg, 72%; colourless oil) was prepared from aldehyde **13** (300 mg, 1.24 mmol) and (*R*)-Alpine-Borane® (0.5 M solution in THF; 7.5 mL, 3.73 mmol) following procedure for (*R*)-**1**. NMR and FT-IR data are identical with (*R*)-**2**. HRMS: for $C_{16}H_{19}D_3O$ calculated 243.2641; found 243.2642.

4.1.15. (1*R*,11*Z*)-[1,10,10-$^2H_3$]-Hexadec-11-en-1-ol [(*R*)-3]. Alcohol (*R*)-**3** (220 mg, 73%; colourless oil) was prepared from aldehyde **14** (300 mg, 1.24 mmol) and (*S*)-Alpine-Borane® (0.5 M solution in THF; 7.5 mL, 3.73 mmol) following the procedure for (*R*)-**1**. ^{1}H NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.1 Hz, CH_3), 1.22–1.39 (18H, m, CH_2[3–9,14,15]), 1.56 (2H, bq, J=7.4 Hz, CH_2[2]), 2.00–2.04 (2H, m, CH_2[13]), 5.32–5.38 (2H, m, CH=CH), 3.62 (1H, tt, J=2×1.5, 2×6.6 Hz, CHD). ^{13}C NMR ($CDCl_3$) δ: 14.00 (q, C_{16}), 22.3 (t, C_{15}), 25.7 (t), 26.5 (tt, J_{CD}=18.7 Hz, C_{10}), 26.9 (t), 29.2 (t), 29.4 (t), 2×29.5 (t), 2×29.6 (t), 32.0 (t, C_2), 32.7 (t), 62.8 (tt, J_{CD}=21.7 Hz, C_1), 129.8 (d, C_{11}), 129.9 (d, C_{12}). FT-IR (CCl_4, cm^{-1}): ν(OH) 3638w; ν(C–OH) 1065m; ν_{AS} (=CH) 3005m; ν(C=C) 1651w; β (=CH) 1413m; γ (=CH) 700w; ν_{AS} (CD_2) 2187w; ν(C–D) 2150w; ν_S (CD_2) 2102w; ν_{AS} (CH_3) 2957s. HRMS: for $C_{16}H_{19}D_3O$ calculated 243.2641; found 243.2640.

4.1.16. (1*S*,11*Z*)-[1,10,10-$^2H_3$]-Hexadec-11-en-1-ol [(*S*)-3]. Alcohol (*S*)-**3** (237 mg, 79%; colourless oil) was prepared from aldehyde **14** (300 mg, 1.24 mmol) and (*R*)-Alpine-Borane® (0.5 M solution in THF; 7.5 mL, 3.73 mmol) following the procedure for (*R*)-**1**. NMR and FT-IR data are identical with (*R*)-**3**. HRMS: for $C_{16}H_{19}D_3O$ calculated 243.2641; found 243.2642.

4.1.17. (1*S*)-Camphanates of alcohols 1–3. Alcohol (30 mg, ca. 125 μmol), (1*S*)-camphanic acid chloride (28 mg, 140 μmol) and anhydrous pyridine (100 μL) in dichloromethane (400 μL) were stirred at rt overnight. The reaction mixture was diluted with ether (10 mL), washed with 0.5 M HCl (2×5 mL), saturated $NaHCO_3$ (2×5 mL), brine (2×5 mL) and dried over $MgSO_4$. Evaporation of the solvents gave an oily residue, which was extracted with hexane. The hexane extracts were combined, evaporated and the oily residue purified by flash chromatography (5% ethyl acetate in light petroleum) to give the ester as a white wax. Yields of camphanates: 90–95%.

4.2. Insects

M. sexta pupae were obtained from the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic) laboratory colony. They were sexed and the female pupae were held in cages at L/D 16:8 photoperiod regime (24–25°C, 40–50% relative humidity) until adults emerged. Virgin females 1 day old were used for experiments. To avoid interference by naturally produced pheromone, in vivo experiments and gland exenterations for in vitro experiments were performed during the photophase, when the female pheromone glands do not produce the pheromone C_{16}-aldehydes.

4.3. In vitro oxidations

The stereochemistry of the alcohol oxidase from *M. sexta* was studied with the intact epidermis of the abdominal tips of females remaining after removing the muscles and all other internal tissues. The epidermis from a single female was used for each experiment. In vitro assays were conducted by incubating the tissue with a deuterated alcohol dissolved in hexane (50 μL of 1 mg mL^{-1} stock solution) and phosphate buffer (5 μL, pH 7.2) in a 2 mL standard vial. After 48 h of shaking (promote the absorbtion of air) at 25°C the tissue was removed from the vial and solution was analysed by GLC–MS (CI). The incubation experiment was repeated three times for each tested compound.

4.4. In vivo oxidations

M. sexta females were anaesthetised with carbon dioxide and their abdomen was gently squeezed by wooden alligator clips until the abdominal tip with pheromone gland extruded.[22] Females were introduced into an anaesthetisation chamber and their pheromone gland was treated twice at interval of 20 min with the solution of 20 μg of labelled metabolic probe (*R*)- or (*S*)-**1** in 1 μL of DMSO.[22] After the solution of metabolic probe was absorbed into the gland (45 min), clips were removed and the moths were returned to their cage. After 6 h, the pheromone glands were excised and extracted with 50 μL of hexane and the extracts were consecutively analysed by by GLC–MS (CI).

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