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BIOSYNTHESIS OF DEFENSIVE ALLOMONES IN LEAF BEETLE LARVAE: STEREOCHEMISTRY OF SALICYLALCOHOL OXIDATION IN *Phratora vitellinae* AND COMPARISON OF ENZYME SUBSTRATE AND STEREOSPECIFICITY WITH ALCOHOL OXIDASES FROM SEVERAL IRIDOID PRODUCING LEAF BEETLES

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Abstract—(7*S*)-[$^2H_5$]-Salicylalcohol (**3**) and (7*R*)-[$^2H_1$]-salicylalcohol (**5**) have been synthesized in order to examine the stereospecificity of salicylalcohol oxidase from the defensive secretion of the salicylaldehyde-producing leaf beetle *Phratora vitellinae*. Oxidation was found to proceed by selective removal of the C(7)-H_R hydrogen atom (*Re*-specificity) to yield salicylaldehyde. (7*S*)-[$^2H_6$]-Benzylalcohol (**9**) was also oxidized *Re*-specifically to benzaldehyde, but in much lower yield, indicating the importance of the orthohydroxy group of salicylalcohol in substrate enzyme binding. The stereospecificities of terpenoid oxidases from six species of iridoid-producing leaf beetle were examined using (1*R*,8*R*)-[$^2H_2$]-8-hydroxygeraniol (**10**), and were all found to oxidize the substrate *Re*-specifically. Cross-activity of oxidation was found in a number of species, with *P. vitellinae* able to oxidize terpenoid (**10**) and two of the iridoid-producing species able to oxidize salicylalcohol analogue (**3**), again with *Re*-specificity. However, when the two substrate analogs were presented together, in equal concentrations, preferential oxidation of the natural analog

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was observed in each case. The kinetics of oxidation for a number of terpenoid and aromatic alcohols by the defensive secretion of the iridoid-producing leaf beetle *Phaedon armoraciae* have been studied, revealing a large difference between the rate of (primary, allylic) terpenoid alcohol oxidation and the rate of salicylalcohol oxidation, thus accounting for the observed selectivity.

Key Words—Salicylalcohol, salicylaldehyde, oxidase, *Re*-specificity, iridoids, leaf beetle, *Phratora*, Chrysomelidae.

INTRODUCTION

Leaf beetles of the family Chrysomelidae are phytophagous insects that form dense, conspicuous aggregates on their host plants, making them particularly vulnerable to predators. In order to counteract this susceptibility, several spectacular defense mechanisms have evolved in the Chrysomelidae, including mechanical, behavioral, and chemical responses (Deroe and Pasteels, 1982). Chemical defense plays a significant role in the protection of both larvae and adults in many leaf beetle species (Pasteels et al., 1982). Larvae of most species in the subtribe Chrysomelina possess nine pairs of defensive glands, located dorsally in the thorax and the first seven abdominal segments (Garb, 1915). The glands are eversible and, when the larva is in a passive state, form reservoirs for the defensive secretion. Upon molestation, however, the everted glands protrude from the body of the insect, exposing a droplet of defensive secretion at their tip.

The chemical composition of larval defensive secretions from several leaf beetle species has been investigated, revealing a range of simple aliphatic and aromatic compounds (Pasteels et al., 1984). Of the 24 species examined to date, 14 contain methylcyclopentanoid monoterpenes (iridoids); seven contain salicylaldehyde and, in some cases, benzaldehyde; one contains a mixture of phenylethyl esters; one possesses a host-plant-dependent mixture of butanoic acid and derivatives (Hilker and Schulz 1994); and one contains the hydroxynaphthoquinone juglone.

The species of leaf beetle that contain iridoids are assumed to synthesize them de novo and, indeed, we now have direct evidence for this (Oldham et al., in preparation). Furthermore, in a recent investigation into iridoid biosynthesis in chrysomelids, we have demonstrated that the methylcyclopentanoids are produced from geraniol, via 8-hydroxygeraniol and 8-oxogeranial, using a pathway similar to that found in plants (Lorenz et al., 1993; Veith et al. 1994). In contrast, those species of leaf beetle that contain salicylaldehyde do not biosynthesize the compound de novo, but obtain it from their host plants (*Populus* or *Salix*) in the form of salicin, a phenolic glycoside, which they cleave and oxidize to give salicylaldehyde (Pasteels et al., 1983). The juglone-producing

species probably also derives its defensive substance from the host plant (*Juglans*), which contains a structurally related phenolic glycoside.

The recent discovery of 8-hydroxygeraniol-8-*O*-β-glycoside in an iridoid-producing leaf beetle (Daloze and Pasteels, 1994) demonstrates that the production of iridoids and salicylaldehyde (and possibly juglone) by leaf beetles, shares two common transformations, namely glucosidation and oxidation. In salicylaldehyde-producing species, a β-glucosidase cleaves salicin to give salicylalcohol (Soetens et al., 1993) and an oxidase converts the diol to salicylaldehyde. Similarly, in the iridoid-producing species, a β-glucosidase is required to hydrolyze 8-hydroxygeraniol-8-*O*-β-glycoside to 8-hydroxygeraniol, which is then oxidized to 8-oxogeranial.

In a previous study, the stereochemical course of 8-hydroxygeraniol oxidation by *Phaedon armoraciae* was investigated, and the oxidation at both C_1 and C_8 was found to be *Re*-specific (Veith et al., submitted). Here we describe an investigation into the stereochemistry of the oxidase in a salicylaldehyde-producing leaf beetle, *Phratora vitellinae*, and compare its stereo- and substrate specificity with those of several leaf beetle iridoid oxidases.

METHODS AND MATERIALS

General

Horse liver alcohol dehydrogenase (HLADH) was purchased from Boehringer (Mannheim, Germany) as a solution (100 mg/10 ml) in KH_2PO_4 buffer (0.02 M, pH 7.0). ^{1}H- and ^{13}C-NMR spectroscopy was conducted on Bruker Cryospec WM 250 and Bruker WM 400 spectrometers. Infrared spectra were measured on a Bruker IFS 88 spectrometer. Gas chromatography–mass spectrometry (GC-MS) was performed on a Carlo-Erba Vega gas chromatograph combined with a Finnigan ITD 800 (ion trap) mass spectrometer in the EI mode (70 eV). CP-SIL-8-CB (Chrompack, 30 m × 0.25 mm) and SE-30 (10 m × 0.32 mm) fused-silica capillary columns were used with helium as the carrier gas (30 cm/sec). Gas chromatography was conducted on a Carlo-Erba HRGC 5300 Mega series gas chromatograph equipped with a SE-30 (10 m × 0.32 mm) fused silica capillary column, with hydrogen as the carrier gas (30 cm/sec), and a FID detector. High-resolution mass spectra (HR-MS) were measured on Finnigan MAT 90 and Kratos MS50 mass spectrometers. Liquid column chromatography was performed on Si 60 (0.040–0.063 mm, E. Merck, Darmstadt, Germany) silica gel.

8-Hydroxygeraniol and (±)-linalool were purchased from Aldrich, geraniol was obtained from Fluka and (±)-citronellol was produced by reduction of (±)-citronellal (Fluka) with $NaBH_4$.

Syntheses

[$^2H_5$]-Salicylaldehyde (2). Sodium (3.5 g, 152 mmol) was slowly added to ice cooled D_2O (8.2 ml) (**caution**) in a 50-ml round-bottomed flask equipped with a reflux condenser. On completion of the reaction, [$^2H_5$]-phenol (**1**) (1 g, 10-mmol) was added to the solution and the reaction mixture heated to 80°C, in order to facilitate the formation of sodium [$^3H_5$]-phenolate. The reaction mixture was then cooled to 65°C, and $CHCl_3$ (1.7 ml, 20 mmol) was added. After 90 min at 65°C, the solution was allowed to cool to room temperature and acidified to pH 1 by addition of 5 N H_2SO_4. The aqueous phase was extracted with diethylether (3 × 20 ml) and the combined organic phase washed with brine (2 × 15 ml) and dried over anhyd. $MgSO_4$. Purification of the crude material was achieved by column chromatography (eluent pentane:diethylether 95:5) to give 400 mg of [$^2H_5$]-salicylaldehyde (**2**) (31% yield). ^1H NMR ($CDCl_3$, ppm): 10.95 (1 H, s); ^{13}C NMR ($CDCl_3$, ppm): 117.24 (t, J = 25 Hz), 119.36 (t, J = 25 Hz), 120.52 (s), 133.30 (t, 2J = 25 Hz), 136.55 (t, J = 24 Hz), 161.66 (s), 196.29 (t, J = 27 Hz); IR (film): 3142, 2123, 1643, 1555, 1394, 1301, 1216, 1162, 1011, 884, 828, 806, 706, 635 cm^{-1}; EI-MS (relative intensity): 127 (M^+, 100), 126 (47), 125 (67), 120 (8), 108 (17), 82 (5), 80 (27), 73 (6), 69 (15), 66 (8), 54 (6), 52 (11), 49 (5), 45 (5); HR-MS: found 127.0682, calculated for $C_7HD_5O_2$ 127.0681.

(7S)-[$^2H_5$]-Salicylalcohol (3). To a solution of [$^2H_5$]-salicylaldehyde (**2**) (50 mg, 0.39 mmol) in 0.1 M sodium phosphate buffer (5 ml, pH 7.5), ethanol (2 ml, 34 mmol), NAD^+ (25 mg, 0.03 mmol), and HLADH solution (500 μl, see General section for details) were added. The reaction was shaken at room temperature for 16 hr before the addition of further 0.1 M sodium phosphate buffer (15 ml, pH 7.5), ethanol (1.5 ml, 25 mmol), NAD^+ (25 mg, 0.03 mmol), and HLADH solution (500 μl). After an additional 12 hr, the characteristic disappearance of color indicated the reduction was complete. The solution was extracted with diethylether (3 × 10 ml), the organic phase washed with brine (15 ml) and dried (anhyd. $MgSO_4$). Column chromatography on silica gel (eluent pentane–diethylether 30:70) afforded 32.7 mg of (7*S*)-[$^2H_5$]-salicylalcohol (**3**) (65% yield). ^1H NMR ($CDCl_3$, ppm): 1.2 (1 H, s), 1.6 (1 H, bs), 4.8 (1 H, s); IR (KBr disk): 3445, 3154, 2928, 2360, 1733, 1599, 1573, 1393, 1250, 1191, 1147, 1006, 934, 884, 799, 722 cm^{-1}; EI-MS (relative intensity): 130 (6), 129 (M^+, 67), 128 (5), 112 (13), 111 (100), 109 (10), 84 (10), 83 (90), 82 (16), 81 (24), 80 (10), 69 (5), 56 (5), 55 (9), 54 (10), 53 (8), 52 (7): HR-MS: found 129.0835, calculated for $C_7H_3D_5O_2$ 129.0838.

(7R)-[$^2H_1$]-Salicylalcohol (5). To a solution of salicylaldehyde (**4**) (35 mg, 0.28 mmol) in 0.1 M sodium phosphate buffer (12 ml, pH 7.5), [$^2H_6$]-ethanol (1.5 ml, 25 mmol), NAD^+ (36 mg, 0.054 mmol), and HLADH solution (600 μl) were added. The reaction was shaken at room temperature for 14 hr before

the addition of further 0.1 M sodium phosphate buffer (1.2 ml, pH 7.5), [$^2H_6$]-ethanol (150 μl, 2.5 mmol), NAD^+ (5 mg, 0.006 mmol), and HLADH solution (70 μl). After 24 hr, additional NAD^+ (5 mg, 7.5 mmol), [$^2H_6$]-ethanol (250 μl, 4.2 mmol), and HLADH (100 μl) were added, and the reaction was allowed to proceed for a further 40 hr. The aqueous phase was extracted with diethylether (3 × 10 ml) and the combined extracts washed with brine (15 ml) and dried (anhyd. $MgSO_4$). Silica column chromatography (eluent pentane–diethylether 30:70) yielded 26.3 mg of (7*R*)-[$^2H_1$]-salicylalcohol (**5**) (75% yield). ^1H NMR ($CDCl_3$, ppm): 2.1 (2 H, s), 4.78 (1 H, s), 6.7–7.2 (4 H, m); ^{13}C NMR ($CDCl_3$, ppm): 64.30 (t, J = 23 Hz), 116.54 (s), 120.09 (s), 124.63 (s), 127.85 (s), 129.52 (s), 156.10 (s); IR (KBr disk): 3448, 3160, 2162, 1615, 1595, 1459, 1387, 1308, 1238, 1191, 1111, 1043, 1008, 939, 898, 864, 847, 750, 727 cm^{-1}; EI-MS (relative intensity): 126 (5), 125 (M^+, 67), 124 (5), 108 (12), 107 (98), 106 (10), 80 (9), 79, (100), 78 (35), 77 (18), 65 (6), 63 (5), 53, (10), 52 (15), 51 (15), 50 (7); HR-MS: found 125.0584, calculated for $C_7H_7DO_2$ 125.0587.

[$^2H_7$]-Benzylalcohol (7). To a stirred suspension of lithium aluminum deuteride (1 g, 23.8 mmol) in diethylether (40 ml), a solution of [$^2H_5$]-benzoic acid (**6**) (2.5 g, 19.5 mmol) in diethylether (30 ml) was slowly added. After 15 min the reaction mixture was hydrolyzed by the addition of water (30 ml) and H_2SO_4 (30 ml, 10%). The phases were separated and the aqueous phase was extracted with diethylether (3 × 20 ml). The combined ether extracts were washed with brine (2 × 30 ml) and dried (anhyd. $MgSO_4$) to give, after evaporation of the solvent, 2.23 g of pure [$^2H_7$]-benzylalcohol (**7**) (99.5% yield). ^1H NMR ($CDCl_3$, ppm): 2.28 (1 H, bs); IR (film): 3344, 2956, 2926, 2854, 2276, 2202, 2113, 2095, 2073, 1698, 1382, 1328, 1250, 1186, 1094, 1051, 1029, 960, 840, 820, 722, 638 cm^{-1}; EI-MS (relative intensity): 116 (7), 115 (M^+, 100), 114 (10), 113 (56), 98 (20), 96 (6), 94 (5), 86 (9), 85 (93), 84 (8), 83 (7), 82 (29), 81 (20), 70 (7), 66 (5), 54 (21), 52 (9), 42 (9); HR-MS: found 115.1006, calculated for C_7HD_7O 115.1015.

[$^2H_6$]-Benzaldehyde (8). To a solution of [$^2H_7$]-benzylalcohol (**7**) (2 g, 17.3 mmol) in dichloromethane (100 ml), manganese dioxide (15.1 g, 173 mmol) was added in portions. After stirring for 24 hr at room temperature, the suspension was filtered through a sinter and the filtrate dried (anhyd. $MgSO_4$). The solvent was removed and the residue purified by silica column chromatography (eluent pentane–diethylether 90:10) to give 1 g of [$^2H_6$]-benzaldehyde (**8**) (50% yield). IR (film): 2281, 2103, 2090, 2066, 2041, 1682, 1630, 1562, 1545, 1376, 1330, 1300, 1179, 1048, 958, 868, 844, 834, 816, 750, 624 cm^{-1}; EI-MS (relative intensity): 113 (7), 112 (M^+, 99), 111 (11), 110 (100), 84 (19), 83 (5), 82 (79), 76 (7), 56 (10), 54 (53), 52 (29), 42 (5); HR-MS: found 112.0777, calculated for C_7D_6O 112.0795.

(7S)-[$^2H_6$]-Benzylalcohol (9). To a solution of [$^2H_6$]-benzaldehyde (**8**)

(1 g, 8.9 mmol) in 0.1 M sodium phosphate buffer (130 ml, pH 7.5), ethanol (40 ml, 675 mmol), NAD^+ (160 mg, 0.25 mmol) and HLADH solution (2 ml) were added. The reaction mixture was shaken at room temperature for 75 min and then extracted with diethylether (3 × 50 ml). The combined ether layers were dried (anhyd. $MgSO_4$). Removal of the solvent gave 770 mg of pure (7*S*)-[$^2H_6$]-benzylalcohol (**9**) (75% yield). ^1H NMR ($CDCl_3$, ppm): 2.02 (1 H, bs), 4.62 (1 H, s): ^{13}C NMR ($CDCl_3$, ppm): 64.8 (t, J = 28 Hz), 126.1–128.4 (5 × s), 140.6 (s); IR (film): 3340, 2900, 2276, 2141, 1415, 1376, 1346, 1306, 1274, 1152, 1057, 1025, 940, 840, 820, 733, 649 cm^{-1}; EI-MS (relative intensity): 115 (6), 114 (M^+, 100), 113 (22), 112 (46), 97 (15), 85 (25), 84 (56), 83 (5), 82 (16), 81 (16), 80 (5), 54 (9); HR-MS: found 114.0936, calculated for $C_7H_2D_6O_2$ 114.0952. $[\alpha]_{546}^{22}$ = + 1.41 (c = 1.0, $CHCl_3$)

Incubation Experiments

Solutions of deuterium-labeled aromatic alcohols **3, 5,** and **9** and labeled 8-hydroxygeraniol (**10**) (100 μl, 0.1% in 0.1 M K_2HPO_4/KH_2PO_4 phosphate buffer, pH 7) were incubated with stock solutions of leaf beetle defensive secretions (100 μl phosphate buffer containing 2–3 insect equivalents per test) overnight. The secretion was collected as described previously (Veith et al., 1996). The aqueous solutions were extracted with ether (200 μl), and the ether layer analyzed by GC and GC-MS (see General section for details) to determine the extent of oxidation of the metabolites. Each incubation was repeated two or three times, depending on the availability of the insects.

Kinetic Experiments with P. armoraciae *Secretion*

A stock solution of *Phaedon* defensive secretion was prepared by collecting the secretions from 24 larvae in sodium phosphate buffer (0.1 M, pH 7, 2 ml). Aliquots of the stock solution (150 μl) were incubated with an equal volume of either 8-hydroxygeraniol, salicylalcohol, geraniol, or citronellol solution (10 mM, in 0.1 M phosphate buffer pH 7 with 1% DMSO). The progress of oxidation was monitored by UV spectroscopy (Perkin Elmer Lambda 2S UV/VIS spectrometer) against the stock solution (150 μl) and phosphate buffer (0.1 M, pH 7, 1% DMSO, 150 μl) at 23°C. Oxidation of 8-hydroxygeraniol and geraniol to 8-oxogeranial and geranial, respectively, was detected at 240 nm, salicylalcohol oxidation to salicylaldehyde was detected at 250 nm, and citronellol oxidation to citronellal was monitored at 300 nm. After 16 hr, incubation was stopped by addition of methanol (50 μl), the products extracted with ethyl acetate (200 μl), and the ratio of alcohol to aldehyde determined by GC analysis. 8-Hydroxygeraniol was incubated with the stock solution of secretion at the beginning of the experiments and at the end in order to account for any decrease in activity of the oxidase over the course of the tests. A repeat experiment, using

an extract of the secretion from an additional 24 larvae (of similar size to the first) gave almost identical results, and the two sets of data were pooled.

Collection and Rearing of Leaf Beetles

Phratora vitellinae, *Gastrophysa viridula*, and *Plagiodera versicolora* were collected near Brussels, Belgium, and near Bonn, Germany; *Phratora laticollis* were collected near Brussels; and *Hydrothassa glabra* and *Prasocuris phellandrii* were collected in Bavaria, Germany. *Phaedon armoraciae* were maintained as a year-round laboratory culture at 18°C, with a 12-hr light–dark schedule, and fed on Chinese cabbage.

RESULTS AND DISCUSSION

Stereochemistry of salicylalcohol oxidation by P. vitellinae. (7*S*)-[$^2H_5$]-Salicylalcohol (**3**) and (7*R*)-[$^2H_1$]-salicylalcohol (**5**) were synthesized enantiospecifically in order to study the stereochemistry of salicylalcohol oxidation by *P. vitellinae*. (7*S*)-[$^2H_5$]-Salicylalcohol (**3**) was synthesized from [$^2H_5$]-phenol (**1**) in two steps (see Figure 1). A Reimer-Tiemann reaction (using NaOD as a base) was employed to convert the phenol (**1**) into [$^2H_5$]-salicylaldehyde (**2**). Reduction of the aldehyde (**2**) to (7*S*)-**3** was achieved using horse liver alcohol dehydrogenase (HLADH) in the presence of ethanol and NAD^+ (for a review of HLADH-catalyzed transformations see Wong and Whitesides, 1994). The enzyme is known to deliver a hydride ion to the *Re*-face of a prochiral aldehyde exclusively, resulting in an (*S*)-absolute configuration at C-7 of alcohol **3.**

(7*R*)-[$^2H_1$]-Salicylalcohol (**5**) was synthesized in one step from salicylaldehyde (**4**) using HLADH in the presence of [$^2H_5$]-ethanol and NAD^+ (see Figure 2). Here, a deuteride ion was delivered to the *Re*-face of aldehyde **4** resulting in the formation of alcohol **5** with (*R*)-absolute configuration at the chiral center.

Incubation experiments, carried out using 0.1% solutions of the chiral salicylalcohols **3** and **5** in phosphate buffer (pH 7.5) together with aliquots of a stock solution of *P. vitellinae* defensive secretion (approximately 2–3 insect equivalents per test), unambiguously demonstrated the stereospecificity of salicylalcohol oxidase in *P. vitellinae*. Salicylalcohol analog **3** was oxidized to salicylaldehyde analog **2** in vitro (see Figure 1) in 91% yield, while alcohol **5** was oxidized to salicylaldehyde **4** (see Figure 2) in 86% yield (due to the large amount of material produced, natural salicylaldehyde from the secretion did not interfere with that formed from in vitro incubation). The identities of the oxidation products were easily confirmed as **2** and **4,** from their mass spectra and GC retention times, since these aldehydes were used as intermediates in the syntheses of the chiral salicylalcohols (see Figures 1 and 2) and were, therefore,

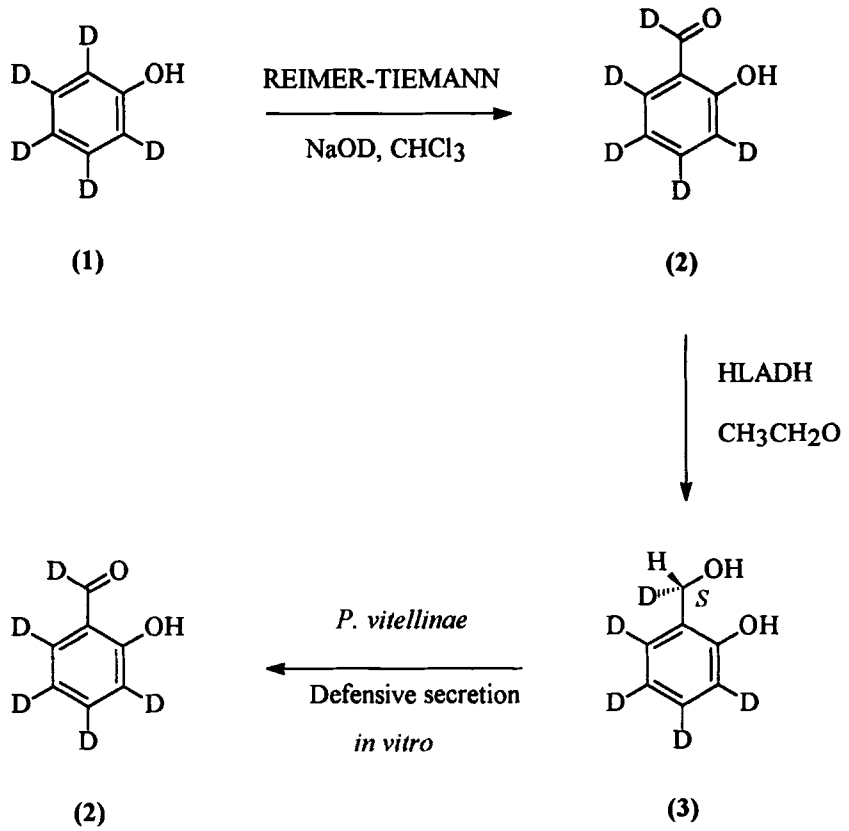


FIG. 1. Synthesis of (7*S*)-[$^2H_5$]-salicylalcohol (**3**), from [$^2H_5$]-phenol (**1**), and its subsequent *Re*-specific oxidation to [$^2H_5$]-salicylaldehyde (**2**) by in vitro incubation with *P. vitellinae* defensive secretion.

already available as standards. Thus, the oxidase in the defensive secretion of *P. vitellinae* has been shown to remove selectively the C-7H_R hydrogen atom of salicylalcohol (*Re*-specificity) during oxidation of the diol to salicylaldehyde.

(7*S*)-[$^2H_6$]-Benzylalcohol (**9**) was synthesized in three steps from [$^2H_5$]-benzoic acid (**6**) (see Figure 3) to provide a metabolic probe that would evaluate the importance of the orthohydroxy group, in salicylalcohol, for substrate-enzyme binding. [$^2H_5$]-Benzoic acid (**6**) was reduced with lithium aluminum deuteride to give [$^2H_7$]-benzylalcohol (**7**). Oxidation of **7** with manganese dioxide afforded [$^2H_6$]-benzaldehyde (**8**), which was reduced with HLADH in the presence of ethanol and NAD^+ to give (7*S*)-[$^2H_6$]-benzylalcohol (**9**) (see Figure 3). In vitro incubation of **9** with *P. vitellinae* defensive secretion gave [$^2H_6$]-

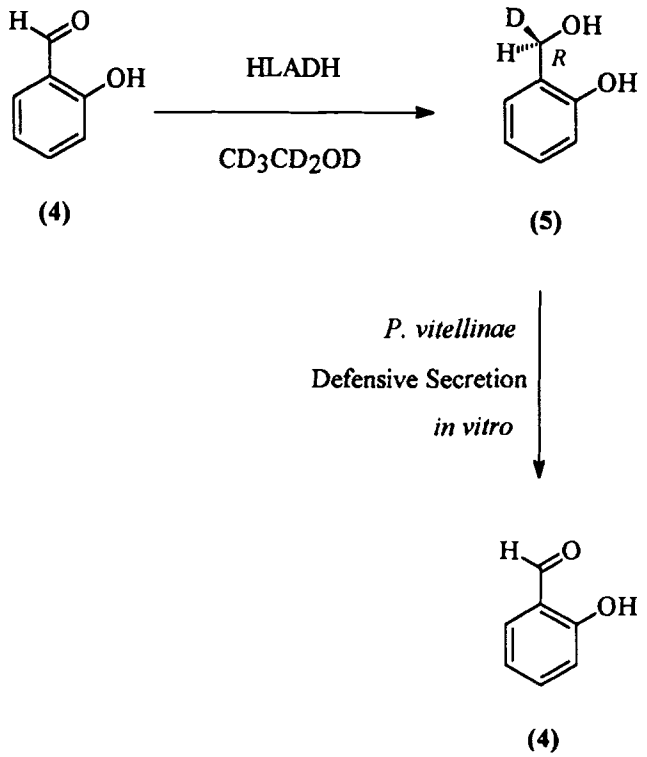


FIG. 2. Synthesis of (7*R*)-[$^2H_1$]-salicylalcohol (**5**) and its *Re*-specific oxidation to salicylaldehyde (**4**) by *P. vitellinae* defensive secretion.

benzaldehyde (**8**) in very low yield (< 1%), identified by comparison of its mass spectrum and GC retention time with those of an authentic sample (used as an intermediate in the synthesis of **9**, see Figure 3). The small degree of oxidation clearly demonstrated that the presence of an orthohydroxy group is important for the interaction of salicylalcohol with the *P. vitellinae* enzyme. However, the absence of a hydroxy group had no effect on the stereospecificity since, as with the salicylalcohols, *Re*-specificity was observed in the oxidation of benzylalcohol (**9**) to benzaldehyde (**8**).

Substrate and Stereospecificity of Oxidases from Chrysomelid Larval Defensive Secretions. In order to compare the stereochemistry of salicylalcohol oxidase from *P. vitellinae* with the terpenoid alcohol oxidases from iridoid-producing leaf beetles, the chiral deuterated salicyl- and benzylalcohols **3**, **5**, and **9**, and chiral deuterated 8-hydroxygeraniol (**10**) (prepared previously, Veith et al., 1996) were incubated with defensive secretions from a number of chry-

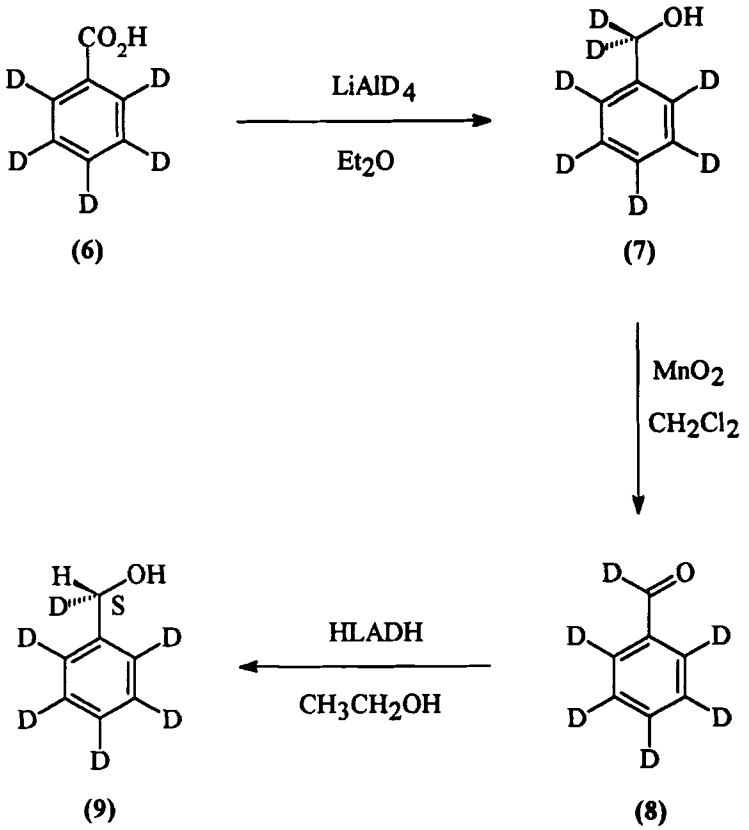
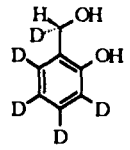
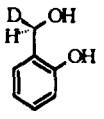
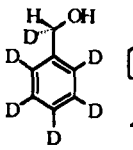
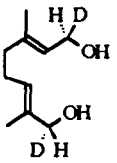


FIG. 3. Synthesis of (*S*)-[$^2H_6$]-benzylalcohol (**9**) from [$^2H_5$]-benzoic acid (**6**) via [$^2H_7$]-benzylalcohol (**7**) and [$^2H_6$]-benzaldehyde (**8**).

somelid species. The results are summarized in Table 1 (due to the limited availability of some species, not all compounds were tested on all species). The observations that the *P. vitellinae* enzyme oxidized **3, 5,** and **9** with *Re*-specificity are reported above. In addition, the secretion from this salicylaldehyde producing species was also found to oxidize [$^2H_2$]-8-hydroxygeraniol (**10**) to 8-oxocitral, again with *Re*-specificity (see Table 1). When (7*S*)-[$^2H_5$]-Salicylalcohol (**3**) and [$^2H_2$]-8-hydroxygeraniol (**10**) were incubated together with the *P. vitellinae* defensive secretion, preferential oxidation of the natural substrate analog **3** was observed (95% oxidation of **3** to **2,** 18% oxidation of **10** to 8-oxocitral, see Table 1). This substrate specificity, together with the identical stereochemistry of both oxidations, suggested that a single enzyme may have been responsible for the oxidation of both alcohols, since a mixture of two oxidases (an

TABLE 1. SUBSTRATE AND STEREOSPECIFICITY FOR OXIDATION OF AROMATIC AND TERPENOID ALCOHOLS BY DEFENSIVE SECRETIONS OF SEVERAL LEAF BEETLE LARVAE

	Substrate				
					
Species of leaf beetle (defensive substance)[a]	(3)	(5)	(9)	(10)	3 + 10
P. vitellinae (SA)	*Re*	*Re*	*Re* (<1%)[b]	*Re*	*Re* (95%) + (18%)[c]
P. armoraciae (IM)	*Re*	*Re*	0	*Re*	*Re* (<1%) + (14%)
P. laticollis (IM)	NT[d]	NT	NT	NT	*Re* (<1%) + (44%)
G. viridula (IM)	0[e]	0	0	*Re*	NT
P. versicolora (IM)	0	0	0	*Re*	*Re* (0%) + (51%)
H. glabra (IM)	NT	NT	NT	*Re*	NT
P. phellandrii (IM)	NT	NT	NT	*Re*	NT

[a]SA = salicylaldehyde, IM = iridoid monoterpene.
[b]Percentage conversion of alcohol to aldehyde.
[c]Individual percentage conversions of alcohols **3** and **10** to their respective aldehydes.
[d]NT = not tested.
[e]0 = no oxidation.

aromatic alcohol oxidase and a terpenoid alcohol oxidase) would be expected to produce a more equal mixture of the two aldehydes. Nevertheless, it was impossible to rule out the presence of two enzymes in very different concentrations (e.g., a high concentration of aromatic alcohol oxidase and a low concentration of terpenoid alcohol oxidase). At this point we do not have sufficient evidence to determine if there are one or several enzymes.

Phaedon armoraciae, an iridoid-producing leaf beetle, possessed a secretion that readily oxidized the natural terpene substrate analog **10** (the resulting acyclic dialdehyde was readily detected and identified by GC-MS as it accumulates in vitro, but is not present in the natural secretion) and the unnatural aromatic alcohol analogs **3** and **5** *Re*-specifically, but which preferentially oxi-

dized the terpene **10** when incubated in the presence of **3** (see Table 1). As with *P. vitellinae*, this suggested a single enzyme was responsible for the oxidation of both alcohols. Larvae of the iridoid-producing leaf beetle *Phratora laticollis* were only available in limited numbers, and their defensive secretion was only incubated with the mixture of **3** and **10.** However, the result of *Re*-specific oxidation of both alcohols, with preference for **10,** demonstrated a similarity of enzyme specificity to that in *P. armoraciae*.

In contrast, the defensive secretions of the iridoid-producing species *Gastrophysa viridula* and *Plagiodera versicolora* only oxidized their natural substrate analog **10** and not the aromatic alcohols **3, 5,** and **9** (see Table 1). However, like all the oxidations, *Re*-specificity was observed, selectively removing H_R from C-1 and C-8 of the substrate. The defensive secretions of *Hydrothassa glabra* and *Prasocuris phellandrii*, two more iridoid-producing leaf beetles, were only incubated with the natural metabolite analog 8-hydroxygeraniol (**10**). *Re*-specific oxidation was also observed in these species.

From the results, it was clear that there were a number of similarities and differences between oxidations carried out by the secretions of various leaf beetle larvae. First, all the leaf beetle secretions oxidized the substrates with *Re*-specificity, demonstrating an identical stereochemical course of oxidation in all species. Secondly, it has been shown that both salicylalcohol oxidase and the various 8-hydroxygeraniol oxidases are oxygen-dependent, $NADP^+$-independent enzymes (Veith et al., 1996; Duffey and Pasteels, in preparation). A major difference between the oxidative capabilities of the secretions was substrate specificity; defensive secretions from *P. vitellinae*, *P. armoraciae*, and *P. laticollis* were all able to oxidize salicyl- and terpenoid alcohols, whereas secretions from *G. viridula* and *P. versicolora* only oxidized their natural terpenoid substrate. Whether this indicates that the former group of species possess two enzymes (a terpenoid alcohol oxidase and an aromatic alcohol oxidase) or whether they each utilize a singe enzyme with relatively low substrate specificity is as yet unclear.

Since the *Phaedon armoraciae* secretion showed interesting substrate tolerance and because it was readily available from our laboratory culture, we studied its ability to oxidize a range of alcohols. A standard stock solution of secretion was prepared by dissolving the defensive secretions from 24 larvae in sodium phosphate buffer (2 ml). Aliquots (150 μl) of the solution were incubated with solutions of 8-hydroxygeraniol, geraniol, citronellol, and salicylalcohol (150 μl, 10 mM). The time course of the oxidations was monitored by UV spectroscopy over 16 hr, and the final concentrations of oxidized product were determined by gas chromatography. In order to account for any deterioration of the oxidase between incubations, 8-hydroxygeraniol oxidation was examined at the beginning of the experiments and again at the end. A reduction in the rate of oxidation (23%) was observed over the six days of measurements, and each

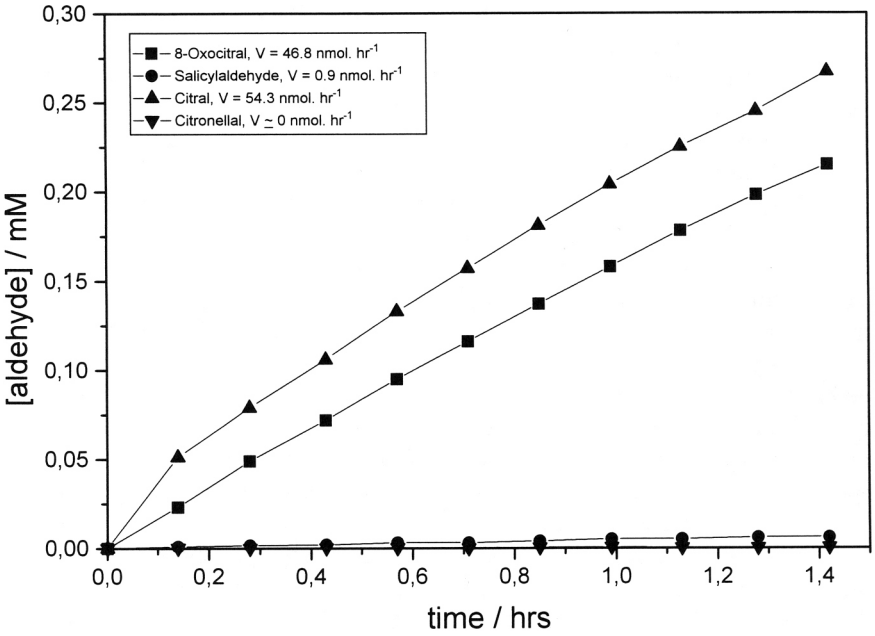


FIG. 4. Plot of aldehyde concentration against time for the oxidation of a number of terpenoid and aromatic alcohols by *Phaedon armoraciae* defensive secretion. Initial reaction velocities (V) are shown in the legend. The data result from the average of two separate sets of incubations.

incubation was corrected accordingly. Figure 4 shows curves of aldehyde (product) concentration vs. time for oxidation of the four alcohols by *Phaedon* defensive secretion (only the initial, largely linear period of the reaction is presented). The curves clearly illustrate that oxidation of 8-hydroxygeraniol and geraniol occurred smoothly. Interestingly, the initial velocity (V) of geraniol oxidation was determined to be slightly greater than that of the natural substrate 8-hydroxygeraniol (V = 54.3 and 46.8 nmol/hr, respectively). Oxidation of salicylalcohol was found to be very slow, with the low concentration of oxidase used (V = 0.9 nmol/hr). This large difference in velocity between 8-hydroxygeraniol and salicylalcohol oxidation explained the substrate specificity observed when the two compounds were incubated together with *Phaedon* defensive secretion (see Table 1). The absence of any detectable citronellol oxidation showed that the *Phaedon* oxidase(s) required an allylic or benzylic alcohol substrate, a result that was also found with 8-hydroxygeraniol oxidase from plants (Ikeda et al., 1991), although the plant enzyme is an $NADP^+$-dependent oxidoreductase, unlike the insect enzyme which is an oxygen dependent oxidase.

In a separate experiment, (±)-linalool (10 mM, 150 μl in buffer/1% DMSO, pH 7) was incubated with the defensive secretion from *Phaedon* (secretion from 2 insects in 150 μl buffer, pH 7) in order to establish whether the oxidase was capable of oxidatively rearranging the tertiary alcohol to geranial. However, no geranial was visible by GC. A positive result may have been significant, since almost all plants contain linalool (normally as a glycoside) and the ability of iridoid-producing leaf beetles to incorporate this substance into the iridoid pathway would have been advantageous to them.

To summarize, salicylalcohol oxidase from *P. vitellinae* is a *Re*-specific oxidase for which the orthohydroxy group of the substrate appears to be important in enzyme-substrate binding. This stereospecificity is identical to that of terpenoid oxidases from a range of iridoid-producing leaf beetle species. The defensive secretion of *P. vitellinae* is capable of oxidizing 8-hydroxygeraniol to 8-oxocitral, just as some of the secretions of the iridoid producers are able to oxidize salicylalcohol. The kinetics of oxidation by the secretion of one such species (*P. armoraciae*) reveal a large difference in reaction velocity between (allylic) terpenoid and aromatic alcohols.

These results suggest either that the oxidases employed by iridoid-producing leaf beetles and by the salicylaldehyde producing species, *P. vitellinae*, are very similar and may share a common origin or that some of the leaf beetles possess both a terpenoid alcohol and aromatic alcohol oxidase. In either case the enzymes link the species. Since it is believed that de novo iridoid production is the more primitive trait (Pasteels et al., 1990), it appears that the salicylalcohol oxidase from *P. vitellinae* may have evolved from an oxidase produced by an iridoid synthesizing ancestor.

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