Generation of Oxidation Artifacts during the Hydrolysis of Norisoprenoid Glycosides by Fungal Enzyme Preparations

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The glycosidic fraction of a *Vitis vinifera* cv. Chardonnay juice was hydrolyzed with three fungal enzyme preparations, i.e., Rohapect C, Rohapect D5L, and Novoferm 12, and also with almond emulsin. The concentrations of nine of the aglycons, all C_{13} norisoprenoids, varied according to the conditions of the hydrolysis. 3-Hydroxy-β-damascone, megastigm-5-en-7-yne-3,9-diol, 3-hydroxy-β-ionone, megastigm-5-ene-3,9-diol, and 3-hydroxy-5,6-epoxymegastigm-7-en-9-one diminished significantly in concentration when the glycosidic fraction was treated with increasing levels of Rohapect C. Loss of these 3-hydroxymegastigmanes was accompanied by the formation of 3-oxo-α-damascone, 3-oxo-β-damascone, and 9-hydroxymegastigma-4,6,7-trien-3-one and an increase in concentration of dehydrovomifoliol. Pure samples of 3-hydroxy-β-damascone and its β-D-glucopyranoside were partially oxidized by Rohapect C to the corresponding oxodamascones. Similar oxidative artifacts were observed when the Chardonnay glycosidic fraction was treated with Rohapect D5L and Novoferm 12. No oxidation was observed with almond emulsin.

INTRODUCTION

Glycoconjugates are increasingly recognized as precursors of flavor in fruits and wines. Consequently, the properties of glycosidases, and the possible application of these enzymes for flavor enhancement, have attracted much interest. Fungal-derived enzyme preparations appear to offer the best combination of glycosidase activities for such applications. These enzymes are commercially available, nonselective with regard to aglycon structure, and relatively tolerant to glucose and have mixed glycosidase functions that are capable of hydrolyzing disaccharide glycosides (Williams, 1991). In using these enzymes, however, high concentrations of the fungal preparation in relation to the substrate are often employed [Drawert et al., 1988 (Pectinol C); Schwab and Schreier, 1988 (hesperidinase); Buttery et al., 1990; Winterhalter et al., 1990 (Rohapect C); Pabst et al., 1991 (Rohapect D5L); Park et al., 1991 (Rohapect 7104)]. High enzyme concentrations are used to ensure complete hydrolysis of all glycosides and to overcome any possible inhibition of the glycosidase activities by phenolic constituents released in the hydrolysis.

The array of products obtained in these enzyme studies is usually considered to reliably depict the aglycon composition, particularly in contrast to the pattern of products furnished by acid hydrolysis. This is based on the assumption that compounds given by glycosidase hydrolysis would not be transformed under the mild conditions of the enzymatic reaction or product isolation.

A recent report of the oxidation of a secondary alcohol to the corresponding ketone, under conditions of glycosidase hydrolysis by a commercial enzyme preparation (Nakanishi et al., 1990), has brought into question the fidelity of products generated in such enzymatic studies. The production of norisoprenoid aglycons without an alcohol function during glycosidase hydrolysis of grape precursor isolates (Sefton et al., 1989; Winterhalter et al., 1990) has prompted us to investigate the possible formation of artifacts in these reactions.

EXPERIMENTAL PROCEDURES

General Procedures. All solvents were of high purity at purchase and were redistilled before use. Analyses were made with a Finnigan TSQ 70 mass spectrometer coupled to a Varian 3400 gas chromatograph. Details of gas chromatography and the isolation of C_{18} reversed phase (RP) Chardonnay juice extracts were as described previously (Sefton et al, 1989; Wilson et al., 1984). The Chardonnay juice was a commercial sample from the 1989 vintage. The synthesis of the β-D-glucopyranoside of 3-hydroxy-β-damascone has been described elsewhere (Skouroumounis, 1991). Three commercial enzyme preparations marketed under the names Rohapect C, Rohapect D5L (Röhm, Darmstadt, Germany), and Novoferm 12 (Novo Ferment, Dittigen, Switzerland) were donated. Almond emulsin (EC 3.2.1.21, 5 units/mg) (Sigma, St. Louis, MO) was available commercially.

Enzymatic Hydrolyses. pH 5 buffer was prepared by dissolving citric acid monohydrate (1 g) and disodium hydrogen phosphate dihydrate (1.868 g) in distilled water (100 mL).

(*a*) *Hydrolysis of 3-Hydroxydamascone β-D-Glucopyranoside.* A solution of the enzyme [Rohapect C (20 or 2 mg) or almond emulsin (20 mg)] in pH 5 buffer (3 mL) was added to a solution of 3-hydroxy-β-damascone-β-D-glucopyranoside (0.5 mg) in pH 5 buffer (3 mL) and the mixture incubated at 37 °C for 16 h. The hydrolyses with Rohapect C were also carried out in nitrogen-sparged solutions under an atmosphere of oxygen-free nitrogen. The hydrolysates were extracted with dichloromethane (3 × 5 mL); the organic solution was washed once with saturated brine (5 mL), dried (magnesium sulfate), and concentrated by evaporation through Fenskes helices prior to analysis by GC–MS.

(*b*) *Treatment of 3-Hydroxy-β-damascone* (**5**) *with Rohapect C.* This was carried out exactly as described for the hydrolysis of 3-hydroxy-β-damascone β-D-glucopyranoside, using 3-hydroxy-β-damascone (0.3 mg) and Rohapect C (20 mg).

(*c*) *Hydrolysis of Chardonnay C_{18} RP Isolates.* A solution of the enzyme [Rohapect C (2, 20, or 200 mg), almond emulsin (100 mg), Novoferm 12 (0.1 or 2.9 mL), or Rohapect D5L (0.1, 1, or 5 mL)] in pH 5 buffer (10 mL) was added to a solution of aqueous C_{18} RP Chardonnay isolate (5 mL, equivalent to 250 mL of juice) in pH 5 buffer (25 mL). The mixture was incubated at 37 °C for 16 h; a solution of 1-octanol (20 μg) in ethanol (100 μL) was then added and the hydrolysate isolated by continuous extraction with dichloromethane over 24 h. The organic extract was dried (magnesium sulfate) and concentrated through Fenskes helices, prior to analysis by GC–MS.

Identification of Aglycons. Compounds **1**–**6** were previously identified as juice components (Sefton et al., 1989). The GC peaks assigned as 3-hydroxy-β-ionone (**7**), megastigm-5-ene-3,9-diol (**8**), and 3-hydroxy-5,6-epoxymegastigm-7-en-9-one (**9**) were symmetrically enhanced by coinjection with authentic samples and had mass spectra identical to those of the authentic samples.

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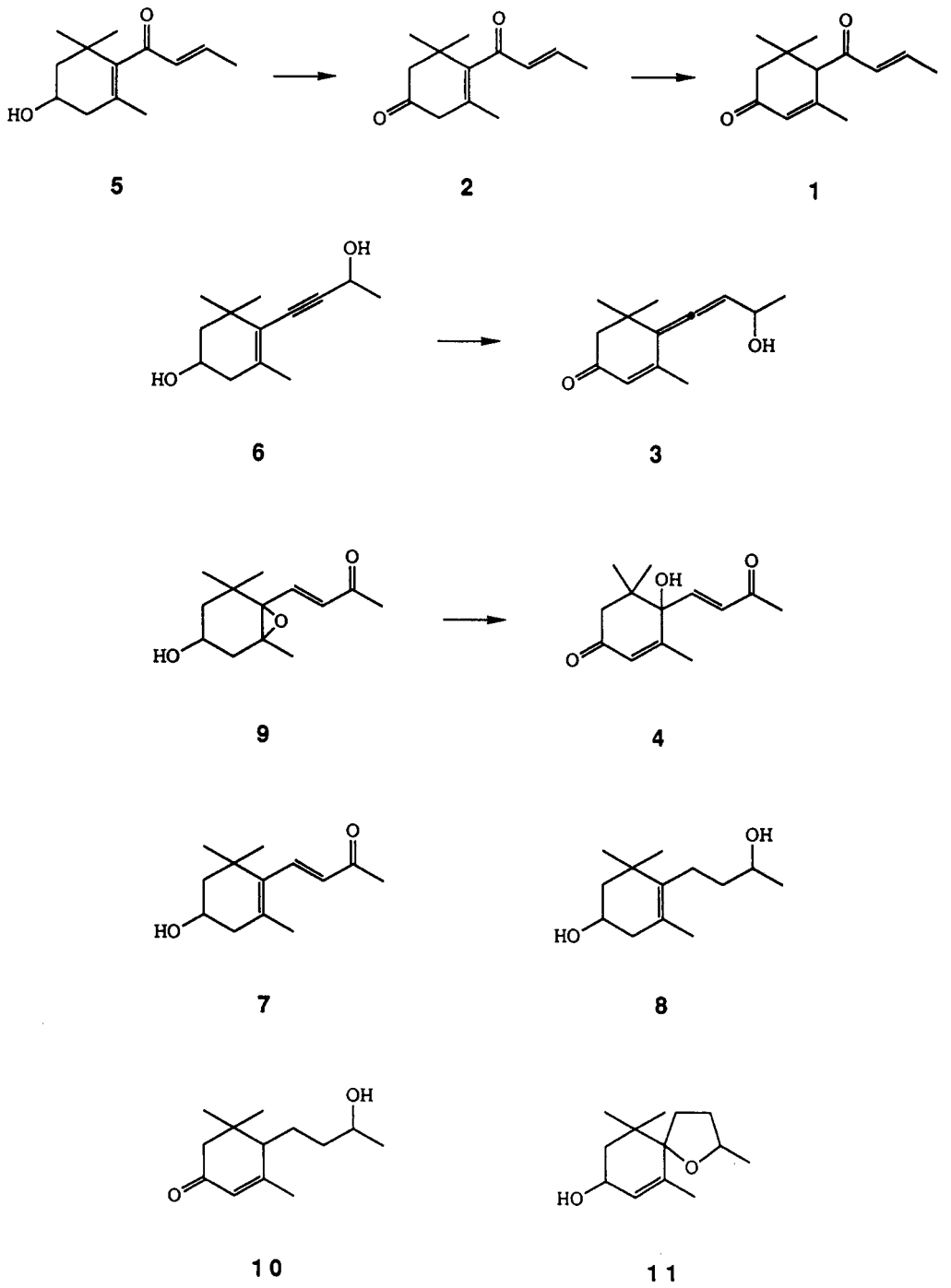

Figure 1. Structures of some aglycons released by enzyme hydrolysis of a Chardonnay juice C_{18} RP isolate.

RESULTS AND DISCUSSION

The oxodamascones 1 and 2 (see Figure 1) have been commonly observed in our laboratory as products from Rohapect C hydrolyses of glycosidic fractions from grape juices. However, these diketones were not observed as free volatiles of grape juices or in volatile fractions released by mild acid hydrolysis of glycosidic precursors. Conversely, 3-hydroxy-β-damascone (5) has been observed as a ubiquitous component of free or acid-generated grape volatiles yet is not always observed as a product in the enzymic hydrolysis of grape C_{18} reversed-phase (C_{18} RP) isolates (Sefton et al., 1989; Winterhalter et al., 1990).

Hydrolysis of a pure synthetic sample of the 3-β-D-glucopyranoside of 3-hydroxy-β-damascone with almond emulsin yielded 3-hydroxy-3-damascone as the sole aglycon. In contrast, hydrolysis of this glucoside with Rohapect C gave the oxodamascones 1 and 2 in addition to the alcohol 5. The ratio of oxidized to nonoxidized products varied according to the amount of the enzyme preparation used in the reaction (see Table I) and was not altered when the enzymatic hydrolysis was carried out under an inert atmosphere. The oxidation products 1 and 2 were also observed, but in a relatively low yield (less than 10%), when unglycosylated 3-hydroxy-β-damascone (5) was treated with Rohapect C.

Table I. Products of Hydrolysis of 3-Hydroxy-β-damascone β-D-Glucopyranoside by Rohapect C

		percentage of product obsd	
	product	with 20 mg of enzyme	with 2 mg of enzyme
1	3-oxo-α-damascone	15	15
2	3-oxo-β-damascone	55	25
5	3-hydroxy-β-damascone	30	60

Table II. Concentrations of Some Compounds Generated by Hydrolysis of a C_{18} RP Chardonnay Juice Isolate with Varying Quantities of Several Enzyme Preparations

	compound	concn, ppb, of compounds obsd						
		with Rohapect C			with Novoferm 12		with Rohapect D5L	with emulsin
		200 mg	20 mg	2 mg	2.9 mL	0.1 mL	0.1 mL	100 mg
1	3-oxo-α-damascone[a]	10	1.5	tr[c]	17	1	20	nd[d]
2	3-oxo-β-damascone	8	nd	nd	14	tr	9	nd
3	9-hydroxymegastigma-4,6,7-trien-3-one	5	tr	nd	5	tr	5	nd
4	dehydrovomifoliol	10	3	2	7	3	10	2.5
5	3-hydroxydamascone[b]	nd	13	12	nd	16	nd	14
6	megastigm-5-en-7-yne-3,9-diol	1	8	6	6	8	1	6
7	3-hydroxy-β-ionone	nd	2	4.5	nd	3	nd	4
8	megastigm-5-ene-3,9-diol	2	10	10	4	16	2	8
9	3-hydroxy-5,6-epoxymegastigm-7-en-9-one	nd	8	8	4	6	nd	7

[a] Cochromatographs with unknown norisoprenoid; approximate concentrations only. [b] Shoulder on peak for 9-hydroxymegastigm-7-en-3-one; approximate concentration only. [c] tr, trace. [d] nd, not detected.

The influence of varying quantities of Rohapect C on the composition of the aglycons released from a C_{18} RP isolate of Chardonnay grape juice was also examined. In these experiments more than 100 aglycons were identified; the majority of these were C_{13} norisoprenoid compounds together with lesser quantities of monoterpenes and shikimate-derived volatiles. Most of these aglycons were formed in similar concentration irrespective of the conditions of the enzymatic hydrolysis, although lower yields of several compounds were observed when lesser concentrations of Rohapect C were employed. Significantly, however, the concentration of nine norisoprenoid aglycons varied according to the hydrolysis conditions, and the variation among some of these compounds appeared to be reciprocal (Table II).

The data in Table II indicate that hydrolysis with the highest concentration of Rohapect C significantly diminished the yield of the 3-hydroxymegastigmanes **5**–**9**, and this decrease appears to be due to oxidation of the 3-hydroxy group in these compounds to a ketone function. It is evident that oxidation of 3-hydroxy-β-damascone (**5**) to the diketones **1** and **2**, already demonstrated for a pure sample of **5** and its corresponding glucoside (Table I), was essentially complete in the experiment using the highest concentration of the fungal enzyme preparation and was minimal at the lowest concentration.

The occurrence of 3-oxo-α-damascone (**1**) can be attributed to a facile rearrangement of the presumed initial oxidation product, i.e., the β-isomer **2** (Sefton et al., 1989). Similarly, oxidation of the hydroxymegastigmanes **6** and **9** was also apparently accompanied by further rearrangement to give more stable end products. Thus, under hydrolysis conditions using the highest concentration of enzyme, the appearance of the allenic ketone **3** and the production of the highest yield of dehydrovomifoliol (**4**) can also be attributed to initial oxidation of the aglycons **6** and **9**, respectively. The latter two 3-hydroxy compounds were found in significant concentration only when small amounts of Rohapect C were employed. Blumenol C (**10**), a likely product of oxidation of the diol **8**, was observed as an abundant aglycon in all of the hydrolysates, but as the former compound cochromatographed with another aglycon, i.e., an isomer of 9-hydroxymegastigma-4,6-dien-3-one, changes in the concentration of **10** could not be determined. Although 3-hydroxy-β-ionone (**7**) was observed only in hydrolyses made with lower concentrations of Rohapect C, no products attributable to its oxidation were identified in the experiment with a high concentration of the enzyme.

The scope of occurrence of these enzyme-induced oxidations was investigated under conditions similar to those employed for the above experiments but substituting for the Rohapect C two alternative fungal enzyme preparations, Novoferm 12 and Rohapect D5L, as well as the plant-derived β-glucosidase almond emulsin (Table II). All of these have been employed in precursor studies (Schwab and Schreier, 1988; Canal Llaubères, 1990; Pabst, et al., 1991).

Novoferm 12 at different concentrations affected the yields of products **1**–**9** in a manner similar to that observed with the Rohapect C. More pronounced oxidation of the grape substrates was observed with Rohapect D5L than with the other enzymes, and even at the lowest concentration used in the study, significant amounts of oxidation products **1**–**4** and concomintant diminutions of alcohols **5**–**9** were observed (Table II).

In contrast to the results obtained with the three fungal enzyme preparations, there was no evidence of oxidation of the glycosidic substrates during hydrolysis with a high concentration of almond emulsion. Indeed, the pattern of products obtained with almond emulsin was similar to that observed in the experiments with the lowest levels of Rohapect C and Novoferm 12, suggesting that at low concentrations fungal enzymes can give products free of artifacts.

All of the norisoprenoids apparently oxidized by these fungal enzymes were 3-hydroxymegastigmanes. Importantly, the side-chain hydroxyls of other megastigmane aglycons and the 3-hydroxyls of bicyclic norisoprenoid compounds such as **11** were not oxidized. Similarly, no oxidation of monoterpene or shikimate-derived alcohols was observed in these experiments.

The 3-oxodamascones **1** and **2** and the allenic ketone **3** are present in the Chardonnay aglycons entirely as artifacts of fungal enzyme oxidation. Dehydrovomifoliol (**4**) appears to be a genuine aglycon; however, increases in concentration of this compound under some conditions of enzyme hydrolysis may also be due to the oxidative artifact. All other 3-oxomegastigmanes, which are the majority of the Chardonnay norisoprenoids, were observed in all enzyme hydrolysates and are presumably genuine aglycons.

The data to hand do not permit an explanation of the mechanism of the oxidation brought about by the fungal enzyme preparations, but it is significant that no volatile products experiencing a reciprocal reduction were observed in the experiments. Also, the transformation into ketones **1** and **2** of the synthetic glucoside of 3-hydroxy-β-damascone, in isolation and either in the presence or in the absence of oxygen, demonstrated that no exogenous cofactor was necessary for the oxidation.

CONCLUSION

Fungal-derived enzymes are the preparations of choice for flavor precursor studies involving glycoconjugates

(Williams, 1991). However, when these products are employed as catalysts in the analysis of aglycons, the possibility of artifact formation must be considered.

The present study demonstrates that when glycosidic conjugates of some 3-hydroxymegastigmanes are subjected to a high concentration of fungal-derived glycosidase enzyme preparation, oxidized aglycon products can be formed as artifacts. Homoallylic, cyclohexanols appear to be particularly susceptible to this oxidation. These substrates are vulnerable to oxidation whether they are reacted upon by the fungal enzyme either in isolation or along with a multitude of other glycosidic derivatives.

Importantly, the ability of fungal enzymes to oxidize these 3-hydroxymegastigmanes is not confined to a single commercially available preparation. At least three enzyme preparations used in precursor studies have been shown to be capable of generating artifacts. The phenomenon may be a characteristic of a larger number of fungal-derived enzymes than was studied here, and indeed it may be general to these preparations and dependent only upon the concentration of fungal enzyme applied to a substrate.

The quantities of enzymes used in these studies were based not on the activities of specific functions, e.g., β-glucosidase, but on the amounts of these preparations used commonly in contempory flavor precursor studies. Presumably, it is the ratio of fungal enzyme to substrate used in precursor hydrolysis that is of importance. Thus, at the lowest concentration applied in the present study, Rohapect C gave only a trace of ketone **1** and otherwise a product distribution similar to that observed with almond emulsin. Apparently, therefore, artifact formation was minimized at this low concentration of Rohapect C.

The use of high levels of fungal enzymes is not always associated with the oxidative loss of 3-hydroxymegastigmanes. For example, hydrolysis of raspberry precursors with Rohapect D5L gave compounds **5**, **7**, and **9** (Pabst et al., 1991), while hydrolysis of apple precursors with hesperidinase yielded 3-hydroxy-β-damascone (**5**) (Schwab and Schreier, 1988). In neither of these studies was the presence of ketones **1**, **2**, or **4** reported, although the ratio of substrate to enzyme used in the experiments was not given.

Complete hydrolysis of all glycosides in a precursor fraction may be slow with a low concentration of added enzyme preparation. It is therefore advisable to carry out these hydrolyses in several experiments, using enzyme preparations at different concentrations. Comparison of the aglycons produced in these experiments will then allow an assessment of those products released slowly and those which may be artifacts.

The observation of artifacts produced by fungal enzymes emphasizes the need to confirm the structures of the glycoconjugates by isolating and characterizing the individual compounds.

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