Biogeneration and Biodegradation of Raspberry Ketone in the Fungus *Beauveria bassiana*

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In growing cultures of the fungus *Beauveria bassiana* (ATCC 7159) the incubation of 4-(4′-hydroxyphenyl)but-3-en-2-one (*p*-hydroxybenzylidenacetone, **3**), of 4-(4′-hydroxyphenyl)butan-2-one (raspberry ketone, **1**), and of the *S* and *R*,*S* forms of 4-(4′-hydroxyphenyl)butan-2-ol (**2**) yields 2-(4′-hydroxyphenyl)ethanol (tyrosol, **4**) as a final product. The experiments support the view that the actual substrate for the Baeyer−Villiger-type degradation is raspberry ketone (**1**) and that there is a kinetic preference in the microbial enzymatic system for the oxidation to **1** of the *S* form of the 4-(4′-hydroxyphenyl)butan-2-ol (**2**).

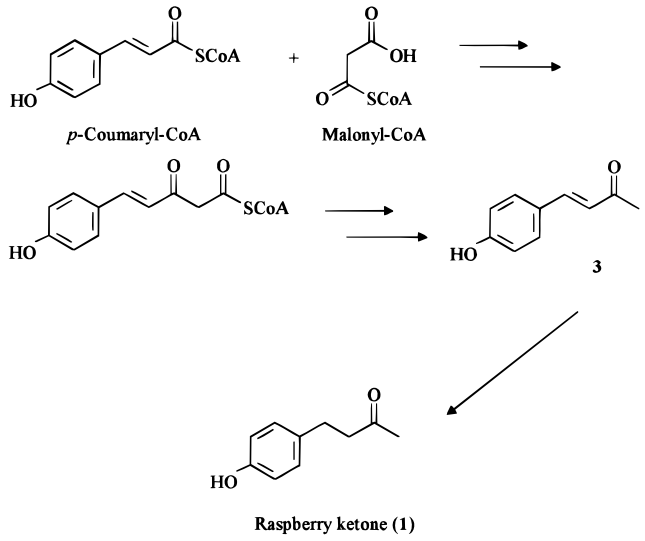
Keywords: *Raspberry ketone; tyrosol; degradation; Beauveria bassiana; Baeyer−Villiger oxidation; stereochemistry*

INTRODUCTION

Raspberry ketone (**1**) entered the reign of flavors in 1918 when it was synthesized by Nomura and Nozawa, in a study designed to evaluate the sensory properties of analogous of zingerone, but, ironically enough, its aromatic significance was not perceived at that time. Its presence in trace amounts in raspberry juice was reported only 40 years later (Schinz and Seidel, 1957, 1961), thus giving origin to the wide diffusion of the synthetic compound as an aroma ingredient in industrial formulations.

Legislative discrimination (*U.S. Code Fed. Regul.*, 1985) between chemically identical food aroma constituents of synthetic origin and those derived from natural sources has stimulated studies designed to obtain substantial amounts of precious flavor materials not accessible through extraction by biotransformation of natural precursors (Stofberg, 1986). Among the aromatic substances for which production through biotechnological methods is highly desirable is raspberry ketone (**1**) (Tiefel and Berger, 1993). In this context, two approaches to the *natural* modification of raspberry ketone (**1**) have recently been reported. The first relies on the enzymatic oxidation of the corresponding 4-(4′-hydroxyphenyl)butan-2-ol (**2**), quite widespread in nature (Parmar et al., 1991), obtained by hydrolysis of the glycoside extracted from *Betula alba* (Dumont et al., 1995). The second (Joulain and Fuganti, 1995; Bosser et al., 1995) involves the bakers' yeast-mediated saturation of the double bond of *p*-hydroxybenzylidenacetone (**3**), which is accessible by condensing 4-hydroxybenzaldehyde of extractive origin with acetone obtained by fermentation. This route imitates the natural biosynthetic process. Indeed, it has recently been shown in raspberry fruit extracts that the late biosynthetic intermediate to rasberry ketone (**1**) is *p*-hydroxybenzylidenacetone (**3**), obtained, in turn, by decarboxylating

Scheme 1. Biosynthetic Pathway of Raspberry Ketone (1) in Raspberry Fruit Showing the Intermediacy of *p*-Hydroxybenzylidenacetone (3) in the Formation of Raspberry Ketone (Borejsza-Wysocki and Hrazdina, 1994)



the intermediate of the condensation of *p*-coumaryl-CoA with malonyl-CoA (Scheme 1) (Borejsza-Wysocki and Hrazdina, 1994).

Proceeding with our studies on the biogeneration of raspberry ketone (**1**) we submitted *p*-hydroxybenzylidenacetone (**3**) to the action of growing cultures of *Beauveria bassiana* (ATCC 7159). This fungus, recently indicated also as *Beauveria sulfurescens* (ATCC 7159) (Taylor, 1970), is well-known for its capacity to reduce selectively double bonds in the presence of other reducible functional groups (Desrut et al., 1981) and widely adopted as an easy to use microbiological oxidant (Floyd et al., 1993). We observed that at short incubation times the *p*-hydroxybenzylidenacetone (**3**) is indeed converted by the latter microorganism into rasberry ketone (**1**) and 4-(4′-hydroxyphenyl)butan-2-ol (**2**), but with further continuation of the incubation with the cells, the only material eventually isolated is tyrosol (**4**). We now present a stereochemical study of the transformation

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of *p*-hydroxybenzylidenacetone (**3**), raspberry ketone (**1**), and (*S*)- and (*R,S*)-4-(4′-hydroxyphenyl)butan-2-ol (**2**) by *B. bassiana* which supports the view that the actual precursor of tyrosol (**4**) is raspberry ketone (**1**).

EXPERIMENTAL PROCEDURES

Synthesis of the Precursors. Raspberry ketone (**1**) was a commercial sample. *p*-Hydroxybenzylidenacetone (**3**) was prepared as described (Nomura and Nozawa, 1918) and recrystallized from toluene. (*R,S*)-4-(4′-Hydroxyphenyl)butan-2-ol (**2**) was obtained by $NaBH_4$ reduction of raspberry ketone (**1**) in ethanol; (*S*)-4-(4′-hydroxyphenyl)butan-2-ol of 86% ee was prepared by bakers' yeast-mediated reduction of raspberry ketone (**1**). To this end, to a stirred mixture of bakers' yeast (2 kg) and D-glucose (500 g) in 4.5 L of tap water was added dropwise a solution of 8 g of raspberry ketone (**1**) in the minimum amount of ethanol within 1 h. After 48 h, the reaction mixture was continuously extracted with ethyl acetate/hexane 8:2. The residue obtained upon evaporation of the dried organic phase was chromatographed on 200 g of SiO_2 (Merck) with increasing amounts of ethyl acetate in hexane, obtaining ca. 4 g of (*S*) 4-(4′-hydroxyphenyl)butan-2-ol (**2**), $[\alpha]^{20}_D$ +16.5° (*c* 1, EtOH); [3,4-$^2H_2$]-4-(4-hydroxyphenyl)butan-2-one (**5**) was obtained upon hydrogenation with deuterium gas (10% Pd/C, ethyl acetate) of *p*-hydroxybenzylidenacetone (**3**): ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.19 (3H, s), 2.75 (1H, m), 2.81 (1H, m), 5.86 (1H, s), 6.78 (2H, d), and 7.04 (2H, d). [1,3-$^2H_5$]-4-(4′-hydroxyphenyl)butan-2-one (**6**) was obtained by submitting raspberry ketone (**1**), 4 g, to the action of 2 g of KOH in 10 mL of deuterated water in 10 mL of 1,4-dioxane at 50 °C for 24 h. The reaction mixture was poured into ice−water/HCl and extracted with ethyl acetate (3 × 150 mL). The residue obtained upon evaporation of the dried organic phase upon crystallization from cyclohexane separates [1,3-$^2H_5$]-4-(4′-hydroxyphenyl)butan-2-one (**6**): ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.8 (2H, s), 5.75 (1H, s), 6.85 (2H, d), and 7.5 (2H, d). 1-Acetate of tyrosol (**4**) was obtained upon 6 h incubation of tyrosol (**4**), 0.7 g (5 mmol), in 40 mL of *n*-hexane and 20 mL of vinyl acetate with 1 g of *Candida cylindracea* lipase (Sigma type VII). The recovery through filtration, evaporation, and short path SiO_2 column chromatography were quantitative. The (GLC) analysis indicated the presence of 1.1% of the diacetate: ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.08 (3H, s), 2.87 (2H, t), 4.24 (2H, t), 5.75 (1H, s), 6.78 (2H, d), and 7.09 (2H, d).

Analytical Procedures. *GLC.* The samples were analyzed by GLC using a 30 m × 0.25 mm i.d. fused silica capillary column (J&W, Folsom, CA) coated with a 0.25 μm layer of cross-linked DB5. Analyses were carried out in a DANI apparatus (Model 8610) equipped with a PTV injector and FID detector. Helium was used as carrier gas at 0.9 bar. The temperature program for all compounds was as follows: 80 °C for 1 min, 10 °C/min, 155 °C for 1 min, 1 °C/min, 165 °C for 2 min, 15 °C/min, 250 °C; total injection, injector temperature 250 °C, detector temperature 250 °C. Retention times were as follows: *p*-hydroxybenzylidenacetone (**3**), 29.39 min; raspberry ketone (**1**), 16.45 min; 4-(4′-hydroxyphenyl)butan-2-ol (**2**), 16.82 min; tyrosol (**4**), 12.65 min.

HPLC. The analyses were performed on a Merck-Hitachi L-6200 apparatus equipped with UV detector L-4200 with a D-2500 integrator. Chiral stationary phase (Chiracel OD, 25 cm × 4 mm, Daicel, Japan) was used for the separation of enantiomers of the acetyl derivatives of (*R,S*)-4-(4′-hydroxyphenyl)butan-2-ol (**2**); the alcohol itself is not separable into its enantiomers with this type of column. The elution conditions were the following: *n*-hexane/*i*-PrOH 9:1, flow 0.6 mL/min, and the detector was set at 220 nm. The retention times were 9.9 and 11.2 min for the *R* and *S* enantiomers, respectively.

Microbial Incubation. Five milliliters of T1 medium was seeded with the microorganism and incubated for 4 days at 30 °C. The biomass was suspended in 4 mL of T3 medium, and 2 mL of this suspension was inoculated in 50 mL of the same medium and shaken at 180 rpm for 24 h at 30 °C. Five milliliters of this culture was inoculated in 50 mL of fresh T3 medium and incubated for 3 days in the same conditions. Three milliliters of the content of the flask was inoculated in 50 mL of MPGB medium and shaken at 180 rpm at 30 °C for 24 h. At this point 50 mg of solid substrates **1**−**3** was added and the mixture stirred at 180 rpm for 48/120 h at 30 °C. The incubation mixture was extracted with 2 × 25 mL of ethyl acetate. The separated organic phase, once dried, was evaporated under vacuum to give a crude extract which was used directly for GLC analysis. Composition of the media: T1, corn step atomized 12 g/L, D-glucose 10 g/L, agar 30 g/L, pH 5.5; T3, bactotryptone 10 g/L, K_2HPO_4 1 g/L, D-glucose 30 g/L, $FeSO_4 \cdot 7H_2O$ 0.01 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $ZnSO_4 \cdot 7H_2O$ 0.3 g/L, KCl 0.5 g/L, pH 7.2; MPGB, D-glucose 20 g/L, peptone 5 g/L, malt 20 g/L.

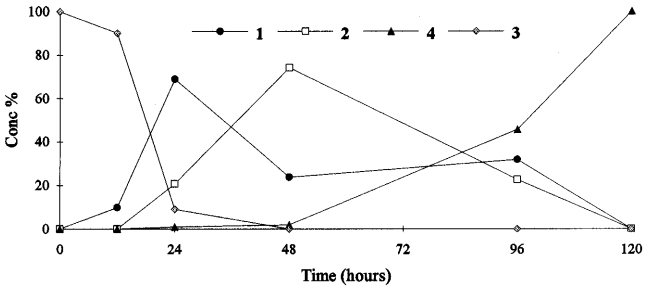


Figure 1. Time course of the biotransformation of *p*-hydroxybenzylidenacetone (**3**) fed on growing cultures of *B. bassiana*. The final biotransformation product, after 120 h of incubation, is tyrosol (**4**).

Deuterated Tyrosol, Samples 7 and 8, from Incubation of Deuterated 5 and 6. From 10 flasks (500 mg of substrate **5** or **6**, 500 mL total volume) of the above culture, ca. 500 mg of crude material, containing less than 1% of the precursors (GLC), was recovered by extraction with 3 × 150 mL of ethyl acetate, after 72 h incubation. Purification on silica gel chromatography of the crude extract gave ca. 300 mg of pure tyrosol. From **5** was obtained **7**: ^{1}H NMR (250 MHz, acetone) δ 2.65 (1H, m), 3.63 (1H, m), 3.87 (1H, OH, m), 6.74 (2H, d), 7.04 (2H, d), and 8.16 (1H, OH, s). From **6** was obtained **8**: ^{1}H NMR (250 MHz, acetone) δ 2.70 (2H, s), 3.58 (1H, OH, s), 6.72 (2H, d), 7.04 (2h, d) and 8.05 (1H, OH, s). To assess the mode of labeling of **7** and **8**, the ^{1}H NMR analyses were also performed on the diacetyl derivatives. Diacetyl derivative of **7**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.04 (3H, s), 2.29 (3H, s), 2.94 (1H, m), 4.27 (1H, m), 7.01 (2H, d), and 7.22 (2H, d). Diacetyl derivative of **8**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.06 (3H, s), 2.29 (3H, s), 2.94 (2H, s), 7.02 (2H, d), and 7.22 (2H, d).

RESULTS AND DISCUSSION

Thus, incubation experiments were performed at 1 g/L for 48−120 h using, respectively, as substrates *p*-hydroxybenzylidenacetone (**3**), raspberry ketone (**1**), and (*S*)-4-(4′-hydroxyphenyl)butan-2-ol (**2**), obtained in the bakers' yeast reduction of **1** and the *R,S* modification of **2** prepared by $NaBH_4$ reduction of **1**. The product distribution in the four experiments, determined by GLC, at the indicated time intervals is reported in Figures 1−4. Inspection of Figure 1, relative to the incubation of *p*-hydroxybenzylidenacetone (**3**) indicates that within the first 48 h, raspberry ketone (**1**) is formed together with 4-(4′-hydroxyphenyl)butan-2-ol (**2**). The capacity of the microorganism of reducing the carbonyl function of **1** is dramatically enhanced when most of the unsaturated substrate has been consumed. However, after 48 h, the amount of alcohol present decreases, while a new educt, clearly identified as tyrosol (**4**), appears. In the meantime, the concentration of raspberry ketone (**1**) slightly increases, but after 120 h, the two intermediates are completely transformed into tyrosol (**4**).

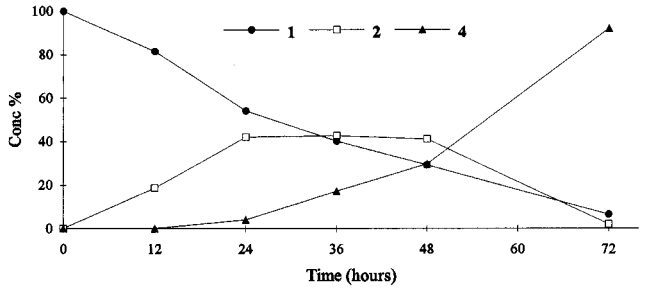


Figure 2. Time course of the biotransformation of the raspberry ketone (**1**) fed on growing cultures of *B. bassiana*. The final biotransformation product, after 72 h of incubation, is tyrosol (**4**).

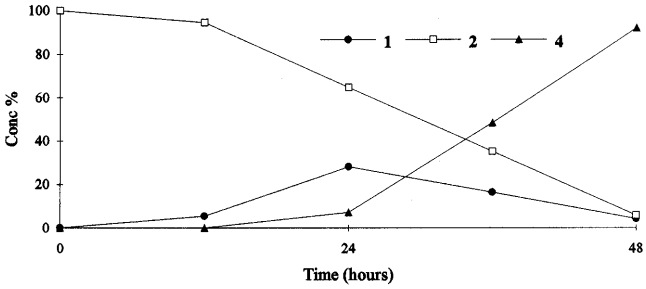


Figure 3. Time course of the biotransformation of (*S*)-4-(4′-hydroxyphenyl)butan-2-ol (**2**) fed on growing cultures of *B. bassiana*. The final biotransformation product, after 48 h of incubation, is tyrosol (**4**).

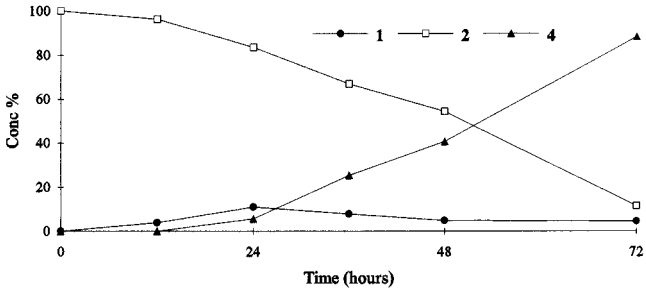


Figure 4. Time course of the biotransformation of (*R,S*)-4-(4′-hydroxyphenyl)butan-2-ol (**2**) fed on growing cultures of *B. bassiana*. The final biotransformation product, after 72 h of incubation, is tyrosol (**4**).

Using raspberry ketone (**1**) as substrate (Figure 2), the phenomenon is quite similar. The formation of tyrosol (**4**) becomes significant after 24 h of incubation, when ca. 50% of the ketone was reduced to the corresponding 4-(4′-hydroxyphenyl)butan-2-ol (**2**). The degradation is complete within 70−80 h. The mode of degradation of the (S) form of 4-(4′-hydroxyphenyl)-butan-2-ol (**2**) outlined in Figure 3 is even more interesting. Indeed, there is an initial oxidation of the carbinol to raspberry ketone (**1**). Tyrosol (**4**) is then formed to a significant extent and the degradation is complete within 50 h. The mode of degradation of racemic 4-(4′-hydroxyphenyl)butan-2-ol (**2**) (Figure 4) is quite similar to that of the *S* enantiomer, with the exceptions that the amount of raspberry ketone actually present in the mixture is lower than in the former case and the time interval at which the degradation is complete rises to 70 h.

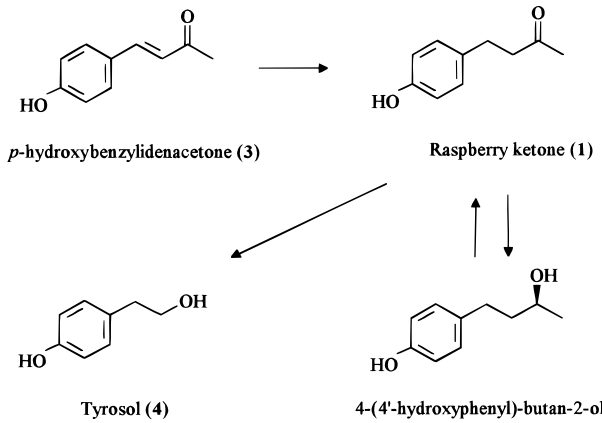
As far as the stereochemical aspects of the above degradation are concerned, the HPLC analysis of the various samples indicates (Table 1) that the 4-(4′-hydroxyphenyl)butan-2-ol (**2**) formed in the incubation of *p*-hydroxybenzylidenacetone (**3**) and raspberry ketone (**1**) after 24 h, when tyrosol **4** is not yet present, holds

Table 1. Stereochemical Course of the Reduction/Oxidation Determined through Feeding Experiments of Raspberry Ketone (1), (*S*)-4-(4′-Hydroxyphenyl)butan-2-ol (2), (*R,S*)-4-(4′-Hydroxyphenyl)butan-2-ol (2), and *p*-Hydroxybenzylidenacetone (3) in *B. bassiana*[a]

substrate	time (h)	**2** (%)	abs config of **2**	ee (%) of **2**
1	0	0		
	12	18.66	*S*	81.0
	24	42.07	*S*	79.0
	36	42.67	*S*	76.6
	48	40.99	*S*	71.4
	72	1.97	*S*	45.0
2 (*S*)	0	100.00	*S*	85.9
	12	94.43	*S*	85.0
	24	64.64	*S*	79.6
	36	35.17	*S*	69.6
	48	8.21	*S*	1.6
2 (*R,S*)	0	100.00	*R, S*	0.0
	12	96.21	*R*	2.0
	24	83.58	*R*	10.8
	36	66.93	*R*	31.8
	48	54.34	*R*	45.4
	72	1.14	*R*	92.3
3	24	20.88	*S*	88.1
	48	74.24	*S*	87.3

[a] In the table are reported only the percentages of the 4-(4′-hydroxyphenyl)butan-2-ol (**2**) obtained, respectively, by reduction of the raspberry ketone (**3**) or by survival from the oxidation of carbinol (**2**).

Scheme 2. Mode of Degradation of *p*-Hydroxybenzylidenacetone (3) in Growing Cultures of *B. bassiana*[a]



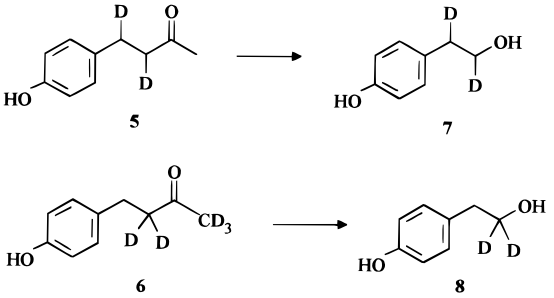
[a] For distribution of the different compounds (raspberry ketone, tyrosol, and carbinol) and for the time of the bioconversion, see Figure 1.

the *S* configuration, with enantiomeric excess (ee) values ranging from 88 to 79%.

However, late in the sequence, when most of the alcohol has been degraded, the residual product still holds the *S* configuration, but with lower ee values. Conversely, in the oxidation of the racemic material **2**, the surviving carbinol becomes slightly enriched in the *R* enantiomer. These observations, seen together, point to a mode of degradation of **1**−**3** illustrated in Scheme 2, with raspberry ketone (**1**), the actual substrate of the oxidation. When the racemic 4-(4′-hydroxyphenyl)-butan-2-ol (**2**) is the substrate, there is a kinetic preference from the microbial oxidizing enzyme(s) for the *S* enantiomer.

Concerning the mechanistic aspects of the process, it seems conceivable that we are dealing with a Baeyer−Villiger-type oxidation of raspberry ketone (**1**), the actual substrate of the process. Indeed, there are examples of this type of degradation in which phenylacetone and long-chain alkyl methyl ketones are the substrates (Walsh and Chen, 1988). However, this type

Scheme 3. Deuterium-Labeled Raspberry Ketone (1) Submitted to the Action of *B. bassiana* To Elucidate the Formation of Tyrosol (4)[a]



[a] The content and the distribution of deuterium in the biotransformed tyrosol are the same of that of the fed raspberry ketone.

of oxidation affords the acetate esters and the same was expected to occur in this case. Since we were apparently analytically unable to detect the presence in the transformation mixtures of this compound, we proceeded to the synthesis of the 1-acetate ester of tyrosol. To distinguish between the two tyrosol (**4**) hydroxyl groups, susceptible to acetylation, we took advantage of the known selectivity of *C. cylindracea* lipase (Pedrocchi-Fantoni and Servi, 1992) in the ester forming/ester hydrolysis reactions. Thus, the 1-acetate of tyrosol obtained by these means (Experimental Procedures) was submitted to the action of *B. bassiana* cultures, finding that its hydrolysis to tyrosol (**4**) is extremely rapid (<30 min). Accordingly, the question of the real intermediacy of the 1-acetate ester in the Baeyer−Villiger-type degradation of raspberry ketone (**1**) to tyrosol (**4**), mediated by *B. bassiana*, is unresolved at present.

Deuterium-labeling experiments further support this view and define the fate of the relevant hydrogen atoms of the substrate **1** during the conversion into tyrosol (**4**). Indeed, when [3,4-$^2H_2$]-raspberry ketone (**5**) was biotransformed into tyrosol, the educt resulted by NMR studies to be labeled as indicated in **7** (Scheme 3). This experiment unequivocally indicates the derivation of tyrosol (**4**) from raspberry ketone (**1**), without changes in the deuterium content of the two methylene groups originally present at positions 3 and 4 of the raspberry ketone framework. Moreover, when raspberry ketone was labeled as indicated in **6**, the derived tyrosol resulted labeled as in **8**, thus supporting an insertion of oxygen into the 2,3-position of the framework of raspberry ketone (**1**) during its conversion into tyrosol (**4**), as occurs in the Baeyer−Villiger degradation (Walsh and Chen, 1988), without change of the oxidation state of the carbon atom ending up at position 1 of tyrosol.

Thus, the present experiments clearly show that in *B. bassiana* the final metabolite of raspberry ketone (**1**) is tyrosol (**4**) through a C-2 chain conceivably shortening via a Baeyer−Villiger oxidation. We will investigate further by means of stereospecifically deuterated substrates the stereochemical changes occurring at position 3 of raspberry ketone (**1**) during its conversion into tyrosol (**4**) and the significance of this observation in relationship with the possible metabolism of raspberry ketone (**1**) in plant and animal systems.

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